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HOUSE FLY NUTRITION

I. QUANTITATIVE STUDY OF THE PROTEIN AND SUGAR REQUIREMENTS OF MALES AND FEMALES^{1,2}

BERNARD GREENBERG³

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TWO FIGURES

The effect of sexual differences in determining nutritional requirements is well demonstrated by mosquitoes and horse-flies. Both males and females of these species feed on nectar and sweet sap; in addition, the females ingest blood to satisfy the protein demands of egg production. That a similar dietary supplement is needed by the female house fly was shown by Glaser ('23) in a qualitative and indirect way. He presented various combinations of sugar and protein to a small number of males and females and observed good egg production only when both classes of food were available. Galun and Fraenkel ('57) measured the average daily consumption of a 5% sucrose solution by equal numbers of males and females kept in a pint jar. This paper reports the consumption of protein and sugar by each sex kept separately under conditions which differ in at least three respects from their experimental procedure: (1) the testing period is considerably longer; (2) the flies are less crowded; (3) the females' ovipositional need is satisfied.

EXPERIMENTAL METHOD

The house flies (*Musca domestica* L.) used in this work have been bred continuously in the laboratory from a strain ob-

¹ University of Illinois, College of Pharmacy, Chicago 12, Illinois.

² Aided by a grant from the University of Illinois.

³ The author gratefully acknowledges the technical assistance of Mr. Paul B. LeVine, a graduate student in the College.

tained in Sheboygan County, Wisconsin, September, 1955. The culture methods have been previously described (Greenberg, '54, '55). Flies for each experiment emerged during a 12-hour period from a jar of CSMA medium directly into a cage, and were fed ad libitum a milk and sugar solution prior to the experiment. The maximum age difference between any two flies was 12 hours. With the use of CO₂ gas, a group of males and females were anesthetized and placed in separate cages during the first 12 hours after emergence. These constituted the virgin flies. The remaining population, approximately equal as to sex, were kept together 6 days to effect copulation, and then separated. The experimental populations, each consisting of 25 flies in an 18-inch cubed cage, were as follows: virgin males; copulated males; virgin females; copulated females. By limiting the number to 25, we hoped to avoid any upset which crowding might produce in the feeding pattern and overall activity of the fly.

The feeding technique described by Dethier and Rhoades ('54) was used except that we employed 10 ml rather than 5 ml volumetric pipettes. The pipettes were selected for uniformity in bore from a single lot of the Kimble "Exax" brand. A line was etched just above the bulb to serve as a reference mark for the original and subsequent filling. The solution was either 5% (w/v) sucrose or 5% (w/v) casein hydrolysate (enzymatic) in tap water, with 0.025% of a U.S.P. formalin solution and a final pH of 6.6. A pipette containing the sucrose solution and another with the protein were placed upright in a pint jar in each cage. The evaporation controls were identically prepared and housed except that these jars were capped with wire mesh to exclude inquisitive flies (fig. 1). Flies fed readily from the tip of the pipette and two or three could be accommodated simultaneously. At the end of each 24-hour period, all pipettes were removed from the cages. By means of a Tuberculin syringe fitted with a no. 27 hypodermic needle the original volume was obtained by returning the meniscus to the etched line. The restored volume of the test pipette

less the restored volume of the evaporation control constituted the 24-hour consumption of that solution by the fly population.

The casein hydrolysate solutions became turbid after 24 hours' exposure and were therefore changed daily. The sugar solutions remained clear but were nevertheless also changed daily. In the first three experiments, the flies had no food for periods of two to three hours each day while the pipettes were read, emptied, washed and dried. Deaths usually occurred dur-

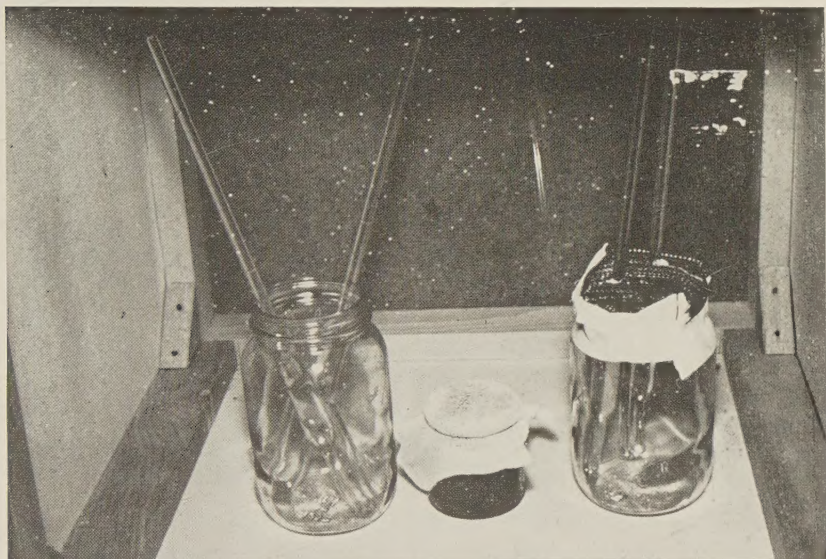


Fig. 1 View of cage showing disposition of pipettes and oviposition jar.

ing these periods and one to three hours after the return of food to the cage. In experiments 4 and 5, this hiatus did not occur. Fractions of fly-days⁴ were used to compute consumption, and the time of death estimated as the average time between two periods of observation.

A 2-oz. specimen jar of CSMA was placed in each cage to provide a suitable medium for oviposition. We chose CSMA which had been used for rearing maggots and was moist and rather odoriferous. The jar was filled to within 1/8" of the

⁴ 1 fly-day = 1 fly living 24 hours.

rim and covered with 8 layers of cheese cloth. The cloth was an effective barrier to feeding but not to oviposition (fig. 2); females laid eggs readily in the top $\frac{1}{4}$ " of the medium. The jars were removed daily, examined for eggs and returned with fresh medium and cover. Cages were kept with the sleeve side up to ease the transfer of jars and pipettes. The

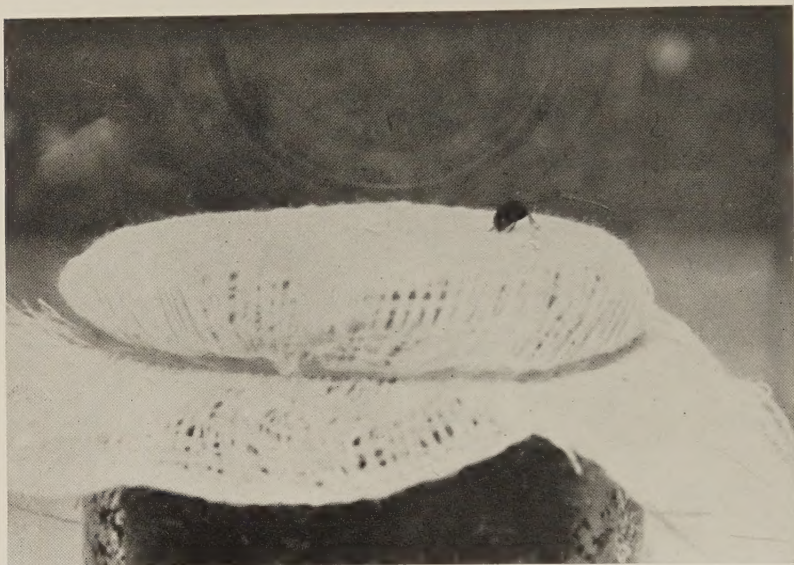


Fig. 2 Fly in the act of ovipositing.

experiments were conducted in an insectary with a temperature of 25° to 26°C and a relative humidity of 40 to 50%. Illumination was provided by daylight fluorescent lights automatically set for the hours between 6 A.M. and 9 P.M.

RESULTS

Table 1 summarizes the data from 4 experiments on protein consumption; table 2 is an analysis of variance based on these data. As shown, the protein uptake of egg-producing female house flies is significantly higher than that of virgin females or males. The overall mean ($\bar{Y}$) for the daily consumption of casein hydrolysate solution by fertile females is 2.2×10^{-3} ml,

or 110 μg actual substance. Virgin females and males are the same with respect to protein consumption; $\bar{Y}$ is 0.8×10^{-3} ml, or 41 μg per fly per day. No significant differences were found between virgin and copulated males in their consumption of protein or sugar.

Table 3 summarizes the data on sucrose consumption. No significant differences were found in sucrose uptake in

TABLE 1
*Summary of protein consumption data*⁵
(all entries $\times 10^{-3}$)

GROUP	DAYS	MEAN	95% CONFIDENCE LIMITS OF THE MEAN
		ml	
Experiment 1			
A ₁ : Copulated ♀	13	2.31	1.21 and 3.40
A ₂ : Copulated ♀	12	2.33	1.38 and 3.28
B: Virgin ♀	13	0.99	0.35 and 1.63
C: Copulated ♂	13	1.09	0.53 and 1.65
D: Virgin ♂	13	0.31	0.08 and 0.54
Experiment 2			
A ₁ : Copulated ♀	14	2.44	1.65 and 3.23
A ₂ : Copulated ♀	14	2.58	1.96 and 3.20
B: Virgin ♀	14	0.60	0.18 and 1.02
C ₁ : Copulated ♂	14	0.90	0.31 and 1.48
C ₂ : Copulated ♂	14	1.40	0.80 and 1.98
Experiment 3			
A: Copulated ♀	5	1.60	0.61 and 2.58
B ₁ : Virgin ♂	6	0.88	0.30 and 1.47
B ₂ : Virgin ♂	7	0.66	0.18 and 1.14
C ₁ : Copulated ♂	6	0.54	0.14 and 0.93
C ₂ : Copulated ♂	6	0.67	0.42 and 1.75
Experiment 4			
A: Copulated ♀	11	1.97	1.47 and 2.47
B: Copulated ♂	11	0.82	0.46 and 1.18

⁵ The statistical analyses of the data were prepared by Dr. H. C. Batson, Research Associate, State of Illinois Department of Public Health, and Miss Dorothy Geischecher of the same institution. The author is grateful for their contribution.

any experiments with the exception of no. 2. Here, the two groups of copulated females and the group of virgin females differed from each other and from the males. The average ($\bar{Y}$) daily consumption of a fertile female, on the basis of the 5 experiments, is 16.7×10^{-3} ml. The male's intake is 15.0×10^{-3} ml.

Total daily intake for a fertile female is, therefore, 18.9×10^{-3} ml, and for a male, 15.8×10^{-3} ml.

TABLE 2
Analysis of variance of protein consumption

SOURCE OF VARIATION	DEGREE OF FREEDOM	MEAN SQUARES	VARIANCE RATIOS (F)
Experiment 1			
Copulated ♀ vs. other groups	1	35.19	23.20 ¹
Between duplicates, copulated ♀	1	0.004	
Between other groups	2	2.36	1.55 ²
Experimental error	59	1.52	
Total	63		
Experiment 2			
Copulated ♀ vs. other groups	1	40.27	35.70 ¹
Between duplicates, copulated ♀	1	0.10	
Between other groups	2	2.25	1.99 ²
Experimental error	65	1.13	
Total	69		
Experiment 3			
Copulated ♀ vs. other groups	1	3.48	7.42 ²
Between other groups	3	0.12	
Experimental error	25	0.47	
Total	29		
Experiment 4			
Copulated ♀ vs. copulated ♂	1	6.99	16.97 ¹
Experimental error	19	0.41	
Total	20		

¹ $p < 0.001$.

² $p < 0.02$.

³ Not significant, $p > 0.05$.

TABLE 3
Summary of sucrose consumption data
 (all entries $\times 10^{-3}$)

GROUP	DAYS	MEAN	95% CONFIDENCE LIMITS OF THE MEAN
<i>ml</i>			
Experiment 1			
A ₁ : Copulated ♀	13	12.89	10.38 and 15.40
A ₂ : Copulated ♀	12	13.75	11.17 and 16.34
B: Virgin ♀	13	14.19	12.11 and 16.27
C: Copulated ♂	13	13.34	11.41 and 15.27
D: Virgin ♂	13	16.84	14.44 and 19.23
Experiment 2			
A ₁ : Copulated ♀	14	19.16	18.14 and 20.18
A ₂ : Copulated ♀	14	21.24	19.05 and 23.43
B: Virgin ♀	14	18.66	17.15 and 20.16
C ₁ : Copulated ♂	14	12.99	11.47 and 14.52
C ₂ : Copulated ♂	14	13.78	12.44 and 15.12
Experiment 3			
A ₁ : Copulated ♀	5	12.71	5.32 and 20.11
A ₂ : Copulated ♀	5	9.44	5.22 and 13.67
B ₁ : Virgin ♂	6	12.96	8.28 and 17.63
B ₂ : Virgin ♂	7	14.94	9.19 and 20.69
C ₁ : Copulated ♂	6	13.06	9.90 and 16.22
C ₂ : Copulated ♂	6	10.20	6.82 and 13.57
Experiment 4			
A: Copulated ♀	11	24.18	21.75 and 26.61
B: Copulated ♂	11	22.45	20.05 and 24.85
Experiment 5			
A: Copulated ♀	9	19.56	17.79 and 21.33
B: Copulated ♂	9	18.86	16.76 and 20.96

DISCUSSION

During oogenesis, many species of insects utilize protein and lipid reserves accumulated from their larval or nymphal stages. Thus, most adult Lepidoptera simply require a sugar solution such as nectar to fulfill the necessary reproductive and dispersal activities. The ultimate in the direction of re-

stricted adult diet is found in the May-flies, Bot-flies and Warble-flies whose vestigial mouthparts preclude feeding. Quite unlike these insects are the mosquitoes and horseflies mentioned earlier, which generally require a blood meal to provide the amino acids and accessory factors necessary for egg production. Saprophagous insects such as the blowflies undoubtedly obtain protein from excrement and the various breeding media in which they oviposit. The data presented here provide quantitative evidence of the house fly's need for an external source of protein to produce eggs.

The protein intake of males and virgin females is two to three times less than that of fertile females. Since flies have good longevity on a diet of sucrose and water alone, it is unlikely that an important non-reproductive need for protein exists.

The ratio of sucrose to protein consumed is 7 to 1 for fertile females and about 16 to 1 for others. In each case, these ratios underscore the importance of sugar in maintaining the life of an adult fly (Greenberg, in press). The 4th experiment was performed 6 months later than the others, but under the same conditions. Its results support the conclusion that males and females typically do not differ in their consumption of sucrose. A single-choice experiment (no. 5) performed concurrently with no. 4 offers further corroboration. In this regard, the results of the second experiment deviated from the others. We cannot invoke oogenesis as an explanation for the higher intake of all females, since virgin females consumed more sucrose as well. An adequate explanation for this deviation eludes us.

In a previous study (Greenberg, '55), a decline in fecundity with age was noted. During the first third of their adult lives, flies produced 43% of the total eggs, while in the last third, their output fell to 22% of the total. In this study, aging had no effect on appetite; flies up to 20 days of age consumed the same amounts of protein and sugar as they did when younger.

SUMMARY

The following data and conclusions are based on a two-choice feeding experiment in which a 5% casein hydrolysate solution and a 5% sucrose solution are available to groups of virgin males, copulated males, virgin females, and copulated females:

1. An egg-laying female house fly consumes daily an average of 2.2×10^{-3} ml of the protein solution. This is two to three times the intake of a virgin female or male and is quantitative evidence of the fly's need for an external source of protein to fulfill the reproductive function.

2. In 4 of 5 experiments, no significant difference was found in the sucrose consumption of males and females. A female consumes 16.6×10^{-3} ml of the sucrose solution daily, and a male consumes 15.0×10^{-3} ml.

3. The ratio of sucrose to protein consumed is 7 to 1 for egg-laying flies, and about 16 to 1 for the others. These ratios underscore the importance of an extrinsic sugar supply in the life of an adult fly.

4. During the observed period from the 6th to the 20th day of its life, aging has no effect on the fly's intake of sugar and protein.

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THE LUMINESCENT FLASH OF POLYCIRRUS^{1,2}

FRANK H. JOHNSON AND MARY M. JOHNSON

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FOUR FIGURES

Although the histology of *Polycirrus* has been investigated in some detail (Dahlgren, '16; Tanassiicuk, '25; Bonhomme, '47, '52), data on the physiology and biochemistry of luminescence in this marine polychaete, as well as in other luminous worms of the family Terebellidae, are largely lacking. Harvey ('55) lists the luminescent system of *Polycirrus* as extracellular and dependent on oxygen, but negative (Harvey, '26) with respect to a "luciferin-luciferase reaction," wherein by definition light emission occurs on mixing a cooled hot-water extract with a cold-water extract of the photogenic organs. The system is also negative with respect to ATP, since after cessation of luminescence in photogenic tissue that has been ground with sand and water, no light appears on addition of ATP (Nicol, '54).

According to Bonhomme ('52) luminescence of *Polycirrus* involves a slow melting of granules, in one of three types of cells present in the epithelium, to a mass of material which emits light when secreted, the exact moment of luminescence being difficult to determine, but possibly accompanying hydration of granules or their dissolution in sea water. This process "se fait lentement et de facon continue ce qui concorde parfaitement avec le caractère lent, peu intense et continu de

¹ Aided in part by Contract Nonr 1353(00), Project NR 165-253, between the Office of Naval Research and Princeton University. Reproduction in whole or in part is permitted for any purpose of the United States Government.

² Hawaii Marine Laboratory Contribution No. 115.

la lumière émise." The results of the present study, dealing with characteristics of the response to electrical stimulation, show that this worm is capable of emitting a series of rapid flashes, of the order of tenths of a second in duration, in addition to slower, more continuous glows.

Polycirrus sp., used in this study, was encountered on rare occasions associated with specimens of a small Chaetopterus found on coral in shallow waters of Kaneohe Bay near the Hawaii Marine Laboratory. In the dark, luminescence in response to irritation appeared to come only from the tentacles, which extended for as much as 5 cm from a yellow body of about 0.5 cm. The light showed up as slow flashes or glows at various distances from the body, and also in tentacles that had been severed from the body.

For recording luminescence, a specimen was placed in the central chamber of a small lucite container similar to the type designed by Chang ('54), with stimulating electrodes dipping into relatively large pools of sea water in wells on opposite sides of the chamber, the walls of the wells constricting towards the chamber so that communication was through narrow slits in order to realize maximum current density on the specimen. The electrodes were connected to an ordinary inductorium, as source of stimuli, by leads passing through a light-tight brass container for holding the lucite assembly, which could be attached to the window of a brass housing of an RCA 1P21 photomultiplier tube. The signal from the photo tube was amplified and fed simultaneously into an Esterline-Angus recorder and a Du Mont type 340 single beam oscillograph.

RESULTS

In most instances, an unpredictable number of repeated stimuli were needed in order to elicit a luminescent response, and the response itself was largely unpredictable with respect to complexity, intensity, and duration. With the method employed, identical successive stimuli could not be given, but oscillograph records of the stimuli (taken separately) indi-

ated a reasonable uniformity. Most likely, therefore, the great variability in response is attributable to unknown physiological factors in the mechanism of excitation.

In only one of a large number of tests did a response occur on the first of a series of consecutive stimulations, consisting of a quick make and break at intervals of approxi-

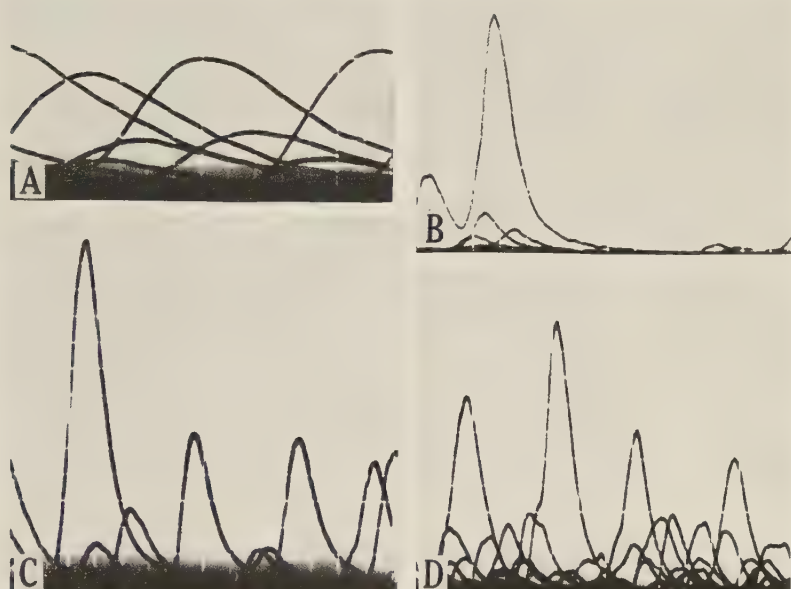


Fig. 1 Luminescent response to single make-break shocks delivered in a sequence at approximately one per second (A and C), and two per second (B and D), with a continuously sweeping oscillograph beam at a frequency of 15 per second in (A), and two per second in (B), (C), and (D). The response first occurred on the 14th consecutive stimulus in (A), on the tenth in (B), principally on the 24th, after very slight responses on the 18th, 19th and 20th in (C), and on the 30th and 40th in (D).

mately one second. A response rarely occurred on the second or third such stimulation; more often it was on about the 10th, and sometimes not until the 40th. Figure 1 (A-D) illustrates the nature of representative responses. Figure 2 shows the 4 most clearly discernible flashes of figure 1 (A) plotted from the oscillograph tracings, arbitrarily taking the same

zero time as that of beginning of response. This time could not be very accurately determined from the record. In figure 2, as plotted however, the time to reach one-half maximum intensity varied from 11 to 12.5 milliseconds, being progressively longer the higher the maximum, and the time for one-half decay varied in a similar manner from 20 to 23.5 milliseconds. The form of the flash in *Polycirrus* resembles that of *Mnemiopsis* (Chang, '54), but these rates of rise and fall of light intensity, in terms of the time to reach half the maximum intensity and to fall to one-half this intensity, respectively, are nearly three times faster at the temperature

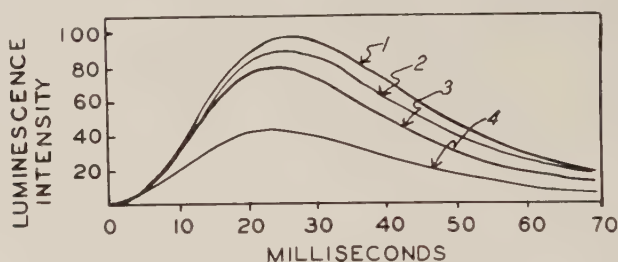


Fig. 2 Four flashes plotted from the oscillograph tracings shown in figure 1 (A), arbitrarily taking the beginning of the response as zero time in each case.

(24–26°C) involved. The rate of decay was exponential as in *Mnemiopsis*, and the same for each of these flashes, as shown by the straight lines with essentially parallel slopes in figure 3, relating the logarithm of decreasing luminescence intensity to time.

In these experiments there was no way to tell from how many, or from what sized, photogenic areas the individual flashes arose, though a solution to this problem could be undertaken with appropriately dissected portions of the tentacles. If the visually observed slow flash, occurring spontaneously or resulting from mechanical stimulation, involves a series of short flashes such as those of figure 1, a fairly large number must take place in the locally luminous area; otherwise, evidence of flicker should become apparent, whereas

none has been reported, nor was any noticed in routine observations in the present study. It would seem more probable that the ordinarily observed response resembles the response to faradic stimulation, as described below.

The results of a series of faradic stimulations of approximately one second duration each, are illustrated in figure 4. Here again, the number of luminescent areas involved is un-

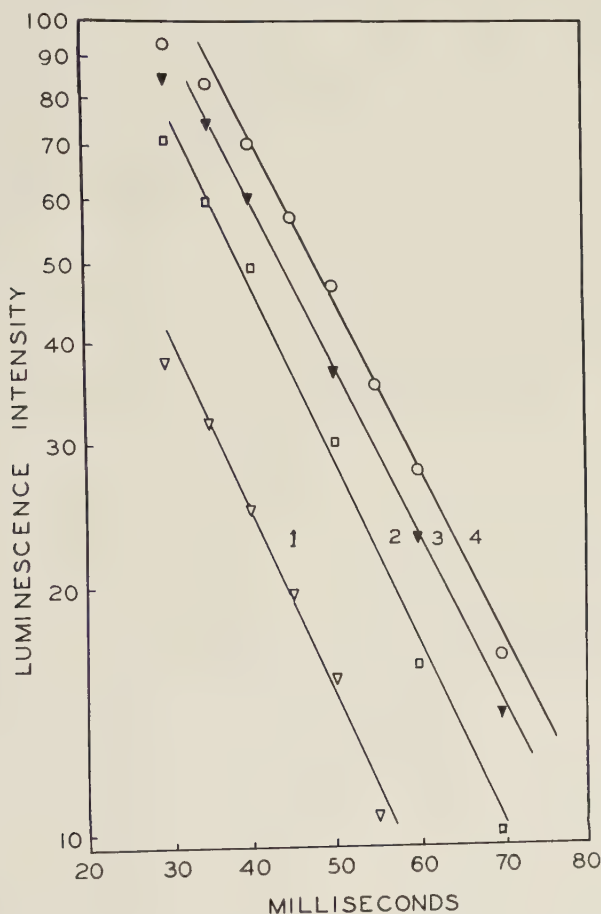


Fig. 3 Rate of decay of luminescence from the maximum, plotted as relative intensity on the logarithmic scale of the ordinate against milliseconds on the abscissa, from the data of figure 2.

fortunately unknown, and it is difficult to tell the precise extent to which the total response consists of summated and fused short flashes. Evidence that short flashes are involved, at least in part, however, is apparent in the wavy character

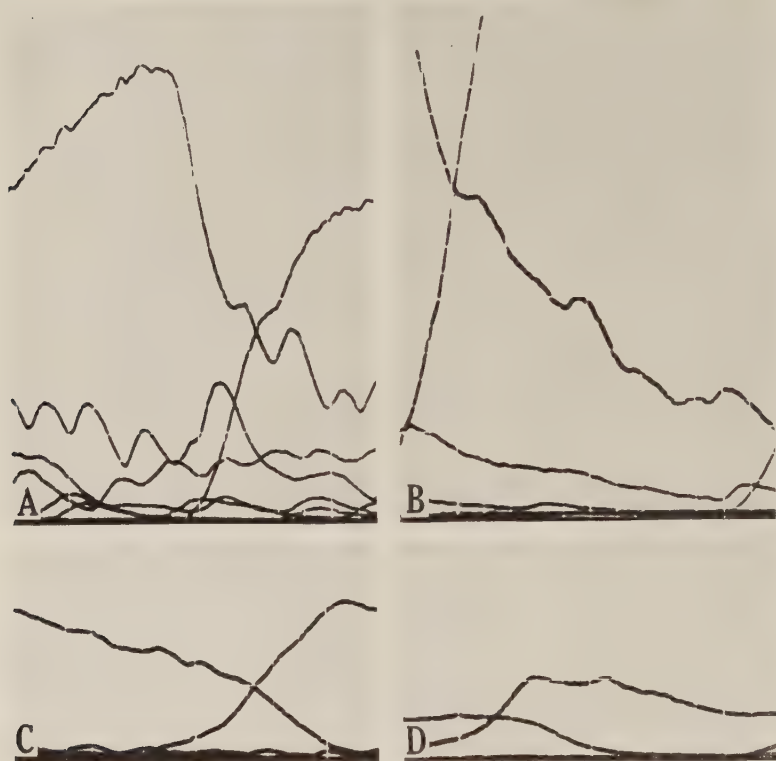


Fig. 4 Responses to 4 consecutive faradic stimulations. A few minutes elapsed between (A) and (B), but (B), (C), and (D) were in close sequence. Amplification of luminescence intensity in (A) and (B) was three times that in (C) and (D). Continuously sweeping oscillograph beam, at a frequency of two per second. Duration of stimulus was approximately 1 sec. for (B), (C), and (D), and several seconds for (A).

of the decay curve in figure 1 A. For the total response, the time to reach half-maximum intensity, as nearly as could be determined from the records, was 132, 166, and 158 milliseconds for figures 1 (A), (C), and (D), respectively, or about

10 times that required in a single, short flash (figs. 1 and 2). Moreover, the maximum tended to be prolonged and the decay slower than exponential.

Some experiments were carried out with respect to the influence of di-isopropylfluorophosphonate (DFP) on the luminescence of this organism, but the oscillograph records were made useless through an error in sweep frequency. The Esterline-Angus records of responses to faradic stimulation of approximately one second's duration indicated that DFP, in a concentration of 0.001 M that reversibly reduces bacterial luminescence (Johnson and Plough, '58), did not abolish the response but made it flatter and more drawn out, with something of an afterglow. Addition of 0.008 M atropin did not counteract this effect. After washing the specimen in several changes of sea water, and allowing repeated 5- to 10-minute rest periods between further faradic stimulations, the sharper form of the flash, without afterglow, again appeared on the Esterline-Angus records, though the maximum intensity which had been generally lower in the DFP solutions, was not regained.

SUMMARY

The luminescent response of *Polycirrus* sp. to repeated inductorium shocks consists of short flashes, of the order of a tenth of a second in duration, with times of slightly over 10 milliseconds to reach half maximum intensity, and 20 to 24 milliseconds for one half decay, as registered on a photomultiplier with oscillograph recorder, at 24 to 26°C. The response rarely occurred on the first stimulus and usually 10 or 12 make-break stimuli at one-second intervals were required, though occasionally as many as forty. The rate of decay from maximum intensity was exponential.

Faradic stimulation elicited a more continuous response, reaching half maximum intensity and one half decay, respectively, in about 10 times the lengths of time required to reach the corresponding points in the short flash. The maximum was broader, and the rate of decay slower than expo-

nential. In some instances evidence of short flashes superimposed on the more gradual course of light intensity was apparent.

In a concentration of 0.001 M, DFP did not abolish the response to short, faradic stimulation, but flattened the course of light intensity versus time, and resulted in an afterglow, according to Esterline-Angus recordings. The DFP effects were not counteracted by 0.008 M atropin, but the more characteristic type of response gradually returned after washing with several changes of sea water.

ACKNOWLEDGMENTS

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THE INFERENCE OF INTRACELLULAR ENZYMATIC PROPERTIES FROM KINETIC DATA OBTAINED ON LIVING CELLS

III. MODE OF GLUCOSE ENTRY INTO THE YEAST CELL-ELABORATION BY MINIMIZED χ^2 ESTIMATION AND URANIUM POISONING

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ONE FIGURE

INTRODUCTION

In a previous paper (Best, '55a), the kinetics of a Michaelis and Menten ('13) enzyme enclosed by a semi-permeable barrier to substrate diffusion was discussed. If v is the rate of utilization, S the external substrate concentration, k and a the Michaelis and Menten parameters of the enzyme *in situ*, and c the membrane desistance (cf. Best, '55), it was demonstrated that

$$a - v = \frac{k}{S/v - c} \quad (1)$$

In those cases in which the primary resistance to the free passage of the substrate from the external medium to the intracellular enzyme is furnished by the cell membrane then (surface area) $\times$ (permeability) $= 1/c$.

A procedure called the "non-linear regression method" was described to permit one to obtain estimates of k , a and c from measurements of utilization rates of a substrate by living cells. This method was applied (Best, '55b) to analyze kinetic data on glucose uptake and sucrose hydrolysis by intact cells of bakers' yeast. Subsequent experience by the author has shown the non-linear regression method depending upon the

minimization of Q (see equation (20) of Best, '55a) to be unreliable, yielding, with certain sets of data, results inconsistent with reality. Since this best fit criterion had no rigorous basis and was merely a common sense attempt to circumvent certain mathematical difficulties, it should be abandoned.

Another defect in the procedure described in the previous papers is that no method of estimating the variance of the parameter estimates was given. The purposes of the present paper are to (1) present a reliable procedure for computation of estimates of a , k and c from kinetic data obtained on intact cell suspensions, (2) derive expressions for computing the variance of these estimates, (3) apply these procedures to data on the uranyl inhibition of glucose uptake of intact yeast cells to elaborate the mechanism of this inhibition.

Heavy metals have been shown to inhibit the penetration of certain non-ionic materials into intact cells. Such studies have recently served as "points of departure" for hypotheses concerning the nature of the cell surface and for speculation regarding the possible role of specialized intra-membrane mechanisms concerned with the transport of specific substances, e.g., hexoses, through the membrane. These studies are of two types both in respect to technique and biological material used: (1) investigations on erythrocytes of various mammalian species with osmotic swelling methods (Jacobs, '50; Jacobs and Corson, '34; Jacobs and Parpart, '37; Jacobs et al., '35, '38; Le Fevre, '47, '48), (2) studies of uranyl and molybdate inhibition of yeast and their relation to enzymatic localities (Rothstein et al., '48a,b, '49; Hurwitz and Rothstein, '51; Barron et al., '48). Since the osmotic swelling equations used in studies of the first type are applicable only if the test material is not metabolized, consideration of substrates normally utilized by the cell in question is excluded.

Studies of Barron et al. ('48) and Rothstein et al. ('48b) indicate the uranyl ion penetrates intact cells of baker's yeast very slowly, if at all, and inhibition of such cells by trace amounts of this ion is produced entirely by its adsorption on the cell surface. Barron believes the inhibitory effect to be

due to a decrease in the membrane permeability produced by relatively non-specific binding of the uranyl ion to the cell membrane. Rothstein et al. believe the effect to be due to inhibition of specific enzyme like cell surface moieties involved in a transmembrane carrier mechanism for glucose.

Some estimation theory

A satisfactory measure of the "goodness of fit" of a theoretical function to a set of experimental points is the statistic χ^2 . Deming ('43) has discussed the general problem of minimum χ^2 regression and the derivation to follow is based on his exposition. Deming's procedure computes the corrections that must be subtracted from a trial set of the parameter values in order to obtain that desired set for which χ^2 is a minimum.

Suppose the error in measuring v is large in comparison to that in S and that v is solved for explicitly as a theoretical function, f , of S and the parameters a , k and c . Denote the j th pair of experimental values of v , S as v_j , S_j . If several, i.e., n_j , replicate measurements of v have been made for S_j then v_j will be their mean. The difference, $F^j = v_j - f(S_j, a, k, c)$, between v_j and the theoretical value given by f will obviously be a function not only of v_j and S_j but also of a , k and c . Trial values of a , k and c shall be denoted as a_0 , k_0 and c_0 and the corrections as A , K and C . Thus

$$a = a_0 - A, \quad k = k_0 - K, \quad c = c_0 - C \quad (2)$$

The value of F^j with the trial values is denoted as F_0^j , its derivatives with respect to a , k and c as

$$F_a^j = (\partial F / \partial a)_j, \quad F_k^j = (\partial F / \partial k)_j, \quad F_c^j = (\partial F / \partial c)_j \quad (3)$$

When a_0 , k_0 and c_0 , with experimental values S_j , are inserted in the expressions for these derivatives, numerical values can be computed for them and F_a^j , F_k^j and F_c^j shall refer to these numerical values. The error variance, σ_j^2 , of v_j will be σ^2/n_j if σ^2 is the variance of the replicate measurements of v . It will

be assumed that σ^2 is approximately the same for all S_j . $N > 3$ different substrate concentrations are used.

F_o^j can be expressed as a power series expansion in A , K and C about F^j . If the trial values are close to those that minimize

$$\chi^2 = \sum_{j=1}^N (F^j)^2 / \sigma_j^2,$$

i.e., a , k and c , then terms involving higher powers of A , K and C may be ignored so $F^j = F_o^j - F_a^j A - F_k^j K - F_c^j C$. Hence χ^2 can be written in the form $\chi^2 = R^2 / \sigma^2$ where

$$R^2 = \sum_{j=1}^N n_j (F_o^j - F_a^j A - F_k^j K - F_c^j C)^2 \quad (4)$$

Since σ^2 is constant, minimization of R^2 will minimize χ^2 . We thus seek those values of the corrections A , K and C giving the minimum value, denoted as $R_{\min}^2$, of R^2 .

From the minimization conditions that

$$\partial R^2 / \partial A = \partial R^2 / \partial K = \partial R^2 / \partial C = 0$$

and dropping the dummy subscript as redundant, one obtains the normal set of equations

$$\begin{aligned} \sum n F_o F_c &= A \sum n F_a F_c + K \sum n F_k F_c + C \sum n F_a F_c \\ \sum n F_o F_a &= A \sum n F_a F_a + K \sum n F_k F_a + C \sum n F_k F_c \\ \sum n F_o F_k &= A \sum n F_a F_k + K \sum n F_k F_k + C \sum n F_c F_c \end{aligned} \quad (5)$$

The symmetric matrix, Φ , of coefficients is defined as

$$\Phi = \begin{bmatrix} \sum n F_a F_a & \sum n F_a F_k & \sum n F_a F_c \\ \sum n F_k F_a & \sum n F_k F_k & \sum n F_k F_c \\ \sum n F_c F_a & \sum n F_c F_k & \sum n F_c F_c \end{bmatrix}. \quad (6)$$

Consider the column comprised of the three terms $\sum n F_o F_a$, $\sum n F_o F_k$, $\sum n F_o F_c$ excluded from Φ . $|\Phi|$ denotes the determinant of Φ , $|A|$ the determinant of the matrix obtained by replacing the first column of Φ by the excluded column, $|K|$ that obtained by replacing the second column, and $|C|$ that obtained by replacing the third column. The estimates for A , K and C , i.e., solution of equations (5), will be

$$A = |A| / |\Phi|, \quad K = |K| / |\Phi|, \quad C = |C| / |\Phi| \quad (7)$$

If Φ^{-1} is the inverse of Φ , then $\Phi^{-1} \sigma^2$ is the variance-covariance matrix of the estimates of A , K and C (cf. p. 55 of

Kempthorne, '52). The variances and covariances of the estimate of A, K and C are also those of a, k and c. Thus

$$\text{Var}(a) = \phi_{aa}^{-1} \cdot \sigma^2, \quad \text{Var}(k) = \phi_{kk}^{-1} \cdot \sigma^2, \quad \text{Var}(c) = \phi_{cc}^{-1} \cdot \sigma^2 \quad (8)$$

where

$$\begin{aligned} \phi_{aa}^{-1} &= \left\{ (\Sigma n F_k F_k) (\Sigma n F_c F_c) - (\Sigma n F_k F_c)^2 \right\} / |\Phi| \\ \phi_{kk}^{-1} &= \left\{ (\Sigma n F_a F_a) (\Sigma n F_c F_c) - (\Sigma n F_a F_c)^2 \right\} / |\Phi| \\ \phi_{cc}^{-1} &= \left\{ (\Sigma n F_a F_a) (\Sigma n F_k F_k) - (\Sigma n F_a F_k)^2 \right\} / |\Phi| \end{aligned} \quad (9)$$

When F^j is randomly distributed about zero, i.e., experimental values differ from theoretical due to random error only, the expected value of χ^2 is the number of degrees of freedom, $N - 3$. Setting χ^2 equal to its expected value one obtains

$$\hat{v}^2 = R_{\min}^2 / (N - 3) \quad (10)$$

as the estimator of σ^2 . Solving equation (1) for v one can write

$$F^j = v_j - \frac{1}{2c} (k + ac + S_j) + \frac{1}{2C} \sqrt{(k + ac + S_j)^2 - 4ac S_j} \quad (11)$$

Taking the partial derivatives with respect to a, k and c, doing considerable algebraic rearrangement, and defining

$$\begin{aligned} X_j &= \frac{4a_0 c_0 S_j}{(\lambda_0 + S_j)^2} & \lambda_0 &= k_0 + a_0 c_0 \\ \psi_{1j} &= \frac{1}{2} (1 - \sqrt{1 - X_j}) & \psi_{2j} &= 1 / \sqrt{1 - X_j} \end{aligned}$$

one finds that

$$\begin{aligned} F_o^j &= v_j - \frac{1}{C_o} (\lambda_0 + S_j) \psi_{1j}, & F_a^j &= C_o F_k^j - \left(\frac{S_j}{\lambda_0 + S_j} \right) \psi_{2j} \\ F_k^j &= \frac{1}{2C_o} (\psi_{2j} - 1), & F_c^j &= (\lambda_0 + S_j) \psi_{1j} / C_o^2 + \frac{a_0}{C_o} F_a^j \end{aligned} \quad (12)$$

Thus, from the trial values a_0 , k_0 , c_0 and the experimental values v_j , S_j , equations (12) can be used to compute F_o^j , F_k^j , F_a^j and F_c^j for every $j = 1, \dots, N$. The various products $n_j F_o^j F_a^j$, $n_j F_a^j F_a^j$, etc. and their sums, $\Sigma n_j F_o^j F_a^j$, $\Sigma n_j F_a^j F_a^j$, etc. can then be computed. These sum values are substituted in determinants $|A|$, $|K|$, $|C|$, and $|\Phi|$, the determinants evaluated and corrections A, K and C obtained. These values are also

substituted into expressions (9) to compute ϕ_{aa}^{-1} , ϕ_{kk}^{-1} , ϕ_{cc}^{-1} . The value of $R_{\min}^2$, for computing $\hat{\sigma}^2$ from expression (10), is calculated from the relation

$$R_{\min}^2 = \frac{\Sigma nF_oF_o + A^2\Sigma nF_aF_a + K^2\Sigma nF_kF_k + C^2\Sigma nF_cF_c + 2AK\Sigma nF_aF_k + 2AC\Sigma nF_aF_c + 2KC\Sigma nF_kF_c - 2A\Sigma nF_oF_a - 2K\Sigma nF_oF_k - 2C\Sigma nF_oF_c}{\Sigma nF_oF_o} \quad (13)$$

Equations (8) can then be used to calculate the variances of a , k and c . As a check on the calculation it should be found that

$$\Sigma nF_oF_o > R_{\min}^2 > 0.$$

Trial values a_o , k_o and c_o can be obtained by the following method. One can verify from equation (1) that v is a monotonically increasing function of S ,

$$\lim_{S \rightarrow \infty} v = a \text{ and } \lim_{S \rightarrow 0} (dv/dS) = a/\lambda \quad (14)$$

Thus, if an "eye fit" curve to a graphical plot of the experimental points is constructed, it will appear as demonstrated in figure 1. The value of v at the point of intersection "X" shall be denoted as v_λ . It can be demonstrated from equation (1) that

$$C = \lambda(2v_\lambda - a)/v_\lambda^2 \quad (15)$$

Trial values of a , k and c are therefore obtained by first making a graphical construction as shown in figure 1, reading off values for a , λ , v and computing c from expression (15). Knowing a , λ and c one can obtain k . The values of a , k and c so obtained are then used as a_o , k_o and c_o respectively for the numerical procedure previously described.

EXPERIMENTAL PROCEDURE

Glass distilled water was used in the preparation of all solutions. Various concentrations of glucose employed were prepared by appropriate dilution from a fresh stock solution prepared gravimetrically from anhydrous glucose and glass distilled water. These solutions were adjusted to pH 3.8 with HCl. Solutions containing uranium were prepared by addition of an accurately measured volume of a freshly prepared uranyl nitrate solution, prior to inoculation with the yeast

suspension. The uranium concentration after inoculation, in those solutions containing uranium, was 0.00357 mg $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ /ml in the case of block 1 and 0.00287 mg/ml in the case of block 2.

Fleischman's baker's yeast, from the interior of a commercial block, was weighed out, washed in glass-distilled water three times by suspension, centrifugation at slow speed, decantation of the supernatant solution and resuspension. The fraction of original yeast weighed out that remained after washing was determined by "hematocrit" measurements on the original and final suspensions. Immediately prior to use for inoculation the pH was adjusted to 3.8 with dilute HCl.

Flasks containing the various glucose solutions were placed on a shaker in a constant temperature bath and inoculated with yeast suspension at zero time. The order in which the flasks of a given block were inoculated and run was randomized with a random number table.

All flasks of a given block were inoculated from the same stock suspension of washed cells within the same day. The suspension used to inoculate the flasks of block 1 was prepared from a different block of commercial yeast at a different time from that used for those in block 2.

At various recorded times after inoculation 5.00 ml aliquots were delivered into test tubes containing 2.00 ml $\text{Ba}(\text{OH})_2$ solution, the tubes stoppered and contents mixed. 2.00 ml ZnSO_4^* were added to each tube, the ppt. removed by centrifugation and supernatant solution samples removed for glucose analysis. These samples were heated in a boiling water bath for 6 minutes to remove volatile reducing materials, cooled and analyzed for glucose by the alkaline ferricyanide ceric sulfate microtitration method of Miller and Van Slyke ('36). After removal of the last aliquot the pH was again measured and if different by more than 0.1 pH unit from 3.8, the run was discarded.

Rates per ml were determined by plotting remaining glucose concentration against time, drawing an "eye fit" curve

to the points and using the zero-time slope as the rate corresponding to the initial glucose concentration.

Data and calculation

Initial utilization rates, v , and concentration, S , of glucose for control and UO_2 -inhibited yeast of each block are given in table 1. These data were used to compute (1) estimates of a , k and c , (2) differences of these estimates between control and UO_2 -inhibited yeast of each block, (3) variances of these estimated differences, (4) tests of significance for the differences.

TABLE 1

Measured rates of glucose uptake per gram of yeast as a function of concentration for uninhibited and UO_2 -inhibited yeast

BLOCK 1				BLOCK 2			
Control		UO_2 -inhibited		Control		UO_2 -inhibited	
S^1	v^1	S	v	S	v	S	v
0.15	0.70	0.15	0.181	0.30	1.08	0.30	0.45
0.30	1.23	0.30	0.619	0.45	1.30	0.45	0.64
0.45	1.80	0.45	0.913	0.60	1.48	0.60	0.87
0.60	2.19	0.60	0.992	0.75	1.60	0.75	1.04
0.75	2.19	0.75	1.18	0.90	1.67	0.90	1.10
0.90	2.36	0.90	1.56	1.20	1.73	1.20	1.46
1.20	2.58	—	—	1.60	1.78	1.60	1.59
1.40	2.58	1.40	2.12	2.00	1.95	2.00	1.63
1.60	2.54	1.60	2.04	2.50	1.87	—	—
2.00	2.60	2.00	2.20	3.00	1.88	—	—
pH = 3.8 Temperature = 25.5°C				pH = 3.8 Temperature = 25.5°C			

¹ S measured in mg glucose/ml; v measured in mg glucose/gm yeast/min.

Trial values a_0 , k_0 and c_0 of a , k and c were obtained by the graphical method described previously and illustrated in figure 1. This was done for each of the four sets of data. In this way¹ one obtains $a_0 = 2.827$, $k_0 = 0.5443$, $c_0 = 0.1608$ and

¹ The trial values shown are written to 4 figures which is, of course, greater than the number of significant figures that can be read from a graph. These values are the means of several independent graphical estimations. Being merely a step in the computation and not the final answer they were not rounded off. In general a single graphical estimate will suffice for the trial values.

$k_o = 0.0898$ as the parameter trial values for the control data of block 1.

These trial values were then used to compute the coefficients of equations (5). In the present data $n_j = 1$ for each j in every set of data. Thus for the control data of block 1

$\Sigma F_o F_a =$	0.207804	$\Sigma F_a F'_c =$	-9.173694
$\Sigma F_o F_k =$	-0.894854	$\Sigma F_k F'_k =$	102.25721
$\Sigma F_o F_c =$	-0.796438	$\Sigma F_k F'_c =$	74.86838
$\Sigma F_a F_a =$	4.762612	$\Sigma F_c F'_c =$	73.92365
$\Sigma F_a F_k =$	-18.250124	$\Sigma F_o F'_o =$	0.050290

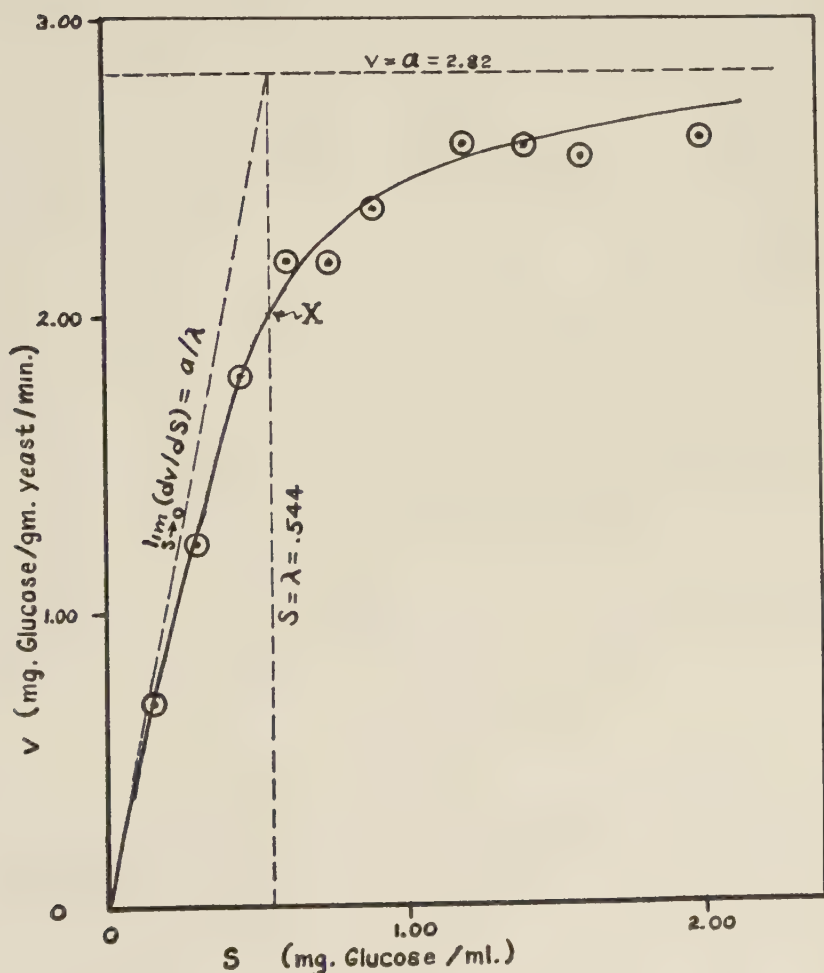


Fig. 1 Trial curve for uninhibited yeast of block 1.

The values of the relevant determinants for this set of data are therefore $|A| = 154.480$, $|K| = 48.5293$, $|C| = -42.3458$ and $|\Phi| = 1147.845$. The values of the corrections are $A = .135$, $K = .0423$ and $C = 0.0369$ hence the corrected estimates of a , k and c are

$$\begin{aligned} a &= 2.827 - 0.135 = 2.692 \text{ mg/gm/min.} \\ k &= 0.0898 - 0.0423 = 0.0475 \text{ mg/cm}^3 \\ c &= 0.1608 + 0.0369 = 0.1977 \text{ gm min./cm}^3 \end{aligned}$$

Using relations (9) one finds $\phi_{aa}^{-1} = 1.7023$, $\phi_{kk}^{-1} = 0.2334$ and $\phi_{cc}^{-1} = 0.13412$. Using expression (13) and the values computed above one finds $R_{\min}^2 = 0.0308$ for the control data of block 1.

In a manner similar to that demonstrated above for the control data, it is found from the UO_2 -inhibited data of block 1 that $a = 2.301$ mg/gm/min., $k = 0.0199$ mg/ml; $c = .5646$ gm min./ml, $\phi_{aa}^{-1} = 3.221$, $\phi_{kk}^{-1} = 0.1026$, $\phi_{cc}^{-1} = 0.1894$ and $R_{\min}^2 = 0.0678$.

By the F ratio test (cf. e.g., Dixon and Massey, '51 or Fisher, '50) it is found that $\hat{\sigma}^2$ is not significantly different for the uninhibited and UO_2 -inhibited data of block 1, hence the $R_{\min}^2$ of the two sets of data can be pooled to obtain an improved estimate of σ^2 . Doing this one finds that $\hat{\sigma}^2 = (R_{\min}^2 + R_{\min}^2)/(N + N' - 6) = 0.00758$. The differences between the parameter estimates for the control and UO_2 -inhibited data of block 1 are 0.391 mg/gm/min., 0.0276 mg/ml and 0.3669 gm min./ml for a , k and c respectively. The estimates of the variances of these differences are

$$\begin{aligned} \text{Var}(a - a') &= (\phi_{aa}^{-1} + \phi_{aa}'^{-1}) \cdot \sigma^2 = 0.0373 \\ \text{Var}(k - k') &= (\phi_{kk}^{-1} + \phi_{kk}'^{-1}) \cdot \sigma^2 = 0.00254 \\ \text{Var}(c - c') &= (\phi_{cc}^{-1} + \phi_{cc}'^{-1}) \cdot \sigma^2 = 0.00245 \end{aligned}$$

each estimate being based on 13 degrees of freedom. The t ratios (see e.g., Dixon and Massey, '51) for each of the differences were constructed. The probabilities (two-tailed test) associated with these t values were found from table 3 of Fisher and Yates ('48) to be 0.065, 0.595 and less than 0.001 for a , k and c respectively.

Data from block 2 were analyzed in a similar manner. For the control of block 2 it was found that $a = 2.017$ mg/gm/min., $k = 0.1522$ mg/ml, $c = 0.122$ gm min./ml, $R_{\min}^2 = 0.0124$ (mg/gm/min.)², $\phi_{aa}^{-1} = 1.347$, $\phi_{kk}^{-1} = 0.940$ and $\phi_{cc}^{-1} = 1.197$, while for the UO_2 -inhibited groups $a = 1.859$, $k = 0.1204$, $c = 0.589$, $R_{\min}^2 = 0.0113$, $\phi_{aa}^{-1} = 3.650$, $\phi_{kk}^{-1} = 1.426$ and $\phi_{cc}^{-1} = 1.445$. Pooling the $R_{\min}^2$ of the control and UO_2 data one obtains $\hat{\sigma}^2 = 0.00198$ based on 12 degrees of freedom. Constructing t ratios for the differences in the parameter estimates between the control and UO_2 data of block 2 one finds the associated probabilities to be 0.15, 0.65 and less than 0.001 for a , k and c respectively.

A combined test of significance for the two blocks can be constructed by the method of Fisher (p. 99, '50). If P_r is the probability for the t ratio of a quantity measured in the r th one of the g independent experiments then

$$\chi^2 = -2 \sum_{r=1}^g P_r$$

with $2g$ degrees of freedom has the sampling distribution of the required combined test statistic. Since there are two blocks, each representing an independent experiment, $g = 2$ in the present case. Doing this combined test one finds for the probability, P , of the difference in a that $0.05 < P < 0.10$, in k that $0.50 < P < 0.70$ and in c that P is very much less than 0.001.

It can be concluded that the observed difference in c between the control and uranyl inhibited yeast is significant while those in a and k are not.

DISCUSSION

The desistance, c , as estimated above is a measure of the resistance to diffusion of the substrate through those barriers interposed between the external medium and the region at which the substrate undergoes its first enzymatic reaction (cf. Best, '55b). If an enzyme-like carrier material at the cell surface is responsible for the transport of hexoses across the

membrane, the present method would yield values of a and k for the hexose carrier reaction and the value of c attributable to stagnant water layers surrounding the cell. Assuming that UO_2 does not penetrate the cell membrane, amounts of the ion inhibitory to glucose utilization would produce a change in a and/or k but not c .

On the other hand, if a and k are the parameters of a reaction in which glucose participates with an enzyme internal to the cell membrane, and therefore not accessible to the uranyl ion, then c should be changed while a and/or k are not. Since, in fact, only c was significantly changed in the uranyl inhibited cells, it is clear that the former hypothesis must be rejected in favor of the latter.

One can validate this view further. Under the first hypothesis the value observed for c would be derived from the concentration gradient of glucose in the external medium in the neighborhood of the cell, being a maximum when no convective mixing occurs to destroy this gradient. If the measured control value of c is significantly greater than this maximum, one must infer a structural barrier interposed between the region of enzymatic reaction and the external medium. For approximately spherical cells this maximum contribution to c , expressed on a weight basis, can be demonstrated (cf. chap. II of Rashevsky, '48; Best, '55a) to be $r_o^2 \rho / 3D$ where ρ is the wet density of the yeast weighed out, D the aqueous diffusion coefficient of the substrate and r_o the cell radius.

For the present purpose one can assume $\rho = 1 \text{ gm/cm}^3$, $D = 3.6 \times 10^{-4} \text{ cm}^2/\text{min.}$ and $r_o < 10^{-3} \text{ cm.}$ Thus $r_o^2 \rho / 3D = 9.3 \times 10^{-4} \text{ gm min./cm}^3$. Using equation (8), the estimates of $R_{\min}^2$, c and ϕ_{cc}^{-1} , one can compute the t ratio for the difference between 9.3×10^{-4} and c . Thus for the two sets of control data one finds, using a single tailed test, that $P = 0.017$ for block 1 and $P < 0.001$ for block 2. Combining these by Fisher's method ('50, p. 99) one finds a resultant $P < 0.001$ that the discrepancies arise from random error. One must conclude the observed values of c for the controls are too large to be

accounted for by external gradients and that a structural barrier exists superficial to the cellular position of the enzyme.

Another suggestion could be made. Disregarding consideration of carrier substances, etc., one might entertain the possibility that the permeability is not concentration independent but decreases with larger glucose concentrations. One might then observe the transport rate tending to plateau asymptotically toward some maximum, however, the interpretation need not necessarily be in terms of an enzymatic carrier. Were this so, over the range of concentrations employed, the values *measured* for a and k , by the *present method*, would depend upon the state of the membrane and be altered in the uranyl inhibited cells. Since this did not happen, c must be independent of glucose concentration over the range of concentrations used in the experiment.

Studies of Derrick et al. ('53) indicate hexokinase of yeast is interior to the cell membrane. So far as is known, the hexokinase reaction is the first undergone by glucose in passing from the medium into the yeast cell. It is reasonable, therefore, to suppose that the values of a and k found in the present study characterize the reaction of glucose with intracellular hexokinase while c is, as initially supposed, actually the distance of the cell surface to glucose. One may conclude that the uranyl ion produces the inhibition of glucose utilization of yeast cells solely by decreasing the permeability of the cell surface to glucose.

Since these results are at odds with some of the conclusions of Rothstein et al. ('48a, '51) a brief comment is in order. Rothstein et al. ('48a) suggest three different types of surface binding sites for UO_2 only one of which is concerned with glucose metabolism. A re-analysis of their data by the present author (Best, '57) however indicates they are dealing with a heterogeneous mixture of binding sites, too numerous in type and effect for resolution by their data. This is reasonable since it is well known that the uranyl ion complexes readily with a variety of materials. The theoretical expressions used by Hurwitz and Rothstein ('51) are not adequate for the

interpretation of the manometric data upon which their kinetic analysis is based, thus the resultant linearity of their Lineweaver and Burk ('34) plots is without simple interpretation.

Another sort of argument has been used by transmembrane carrier theory proponents. Jacobs ('50) estimates only 1 to 0.05% of the human erythrocyte surface is covered by adsorption of Cu ions sufficient to reduce its glycerol permeability by 90%. This result is used to argue against the relevance of the effect, observed by Langmuir and Schaefer ('37), of this ion on fatty acid monolayers and in favor of specific enzyme-like loci concerned with glycerol entry.

Instead of treating the ion as a hard sphere, one should ask the size of the sphere of effective electrostatic influence of a Cu ion in aqueous medium. If the energy of a polar group due to the electrostatic field surrounding the Cu ion is small in comparison to the most probable thermal energy kT , where k is the Boltzman constant and T the absolute temperature, the field can be considered negligible. If this energy is large in comparison to kT , it is not negligible. Consider that sphere in which the field energy is twice kT for a univalent polar group exposed to the field. Let e denote the unit electronic charge, d the dielectric constant of water, and r the distance from the center of the Cu ion. The magnitude of the potential energy of a polar group of charge magnitude e at a distance r will be $\phi = 2e^2/rd$ and we shall desire that value r^* of r for which $\phi = 2kT$. Using the values $e = 4.8 \times 10^{-10}$ e.s.u., $d = 79$, $k = 1.4 \times 10^{-16}$ ergs/°K, $T = 300^\circ\text{K}$ one finds $r^* = 7 \times 10^{-8}$ cm $= 7\text{\AA}$. Jacobs' estimates of the number of Cu ions adsorbed per erythrocyte fall in the range 3×10^7 to 10^6 . Since the human erythrocyte is a biconcave disc of about $7.5\text{ }\mu$ diameter one finds 54 to 1.8% of the erythrocyte surface "blanketed" by the Cu ions.

From the Rothstein et al. ('48a) figure of 10^7 cells/mg yeast, assuming spherical shape and density of 1.0, a mean radius of $2.9\text{ }\mu$ can be computed for the cells used in his experiments. They obtained 100% inhibition with approximately 5×10^7 UO_2 ions adsorbed per yeast cell. Computing as before one

finds that 97% of the cell surface could be "blanketed" by the electrostatic field of the UO_2 ions.

Hence, in both the Cu inhibition of erythrocytes and the UO_2 inhibition of yeast, the proportion of cell surface blanketed by the electrostatic fields of the adsorbed ions is far from negligible and might be as large as 100%. Because of this, it seems premature to discard the possible interpretation suggested by the Langmuir and Schaefer effect.

SUMMARY

The equation describing the kinetics of a Michaelis and Menten ('13) type of enzyme enclosed by a membrane that is semi-permeable to the substrate has been derived previously (Best, '55a; Hearon, '49, p. 85). Such a system is characterized by the membrane desistance, c , and the enzymatic parameters a and k . The desistance is a measure of the resistance to diffusion of the substrate caused by those barriers interposed between the external medium and the cellular location of the first enzymatic reaction undergone by the substrate.

A method is derived for estimating a , k and c from sets of measurements of the rate of substrate utilization as a function of the concentration by minimizing the χ^2 of the fit of the theoretical equation to the experimental points. Expressions are derived for computing the variances of these estimates. This procedure computes the corrections to a set of preliminary trial values of the parameters. A method of obtaining the trial values is given.

Rates of glucose utilization were measured as a function of glucose concentration for uninhibited and uranyl inhibited suspensions of cells of baker's yeast. Estimates of a , k and c for uninhibited and uranyl treated cells were computed from these data by the minimum χ^2 procedure. The variance of the difference of each of these parameters between control and inhibited cells was computed and tests of significance made for the differences. The difference in the desistance, c , was found to be highly significant ($P < 0.001$) while those in the enzymatic parameters a and k were not. The entire in-

hibitory effect of the uranyl ion was therefore attributable to the observed increase in c . The values of c measured for the uninhibited cells are significantly larger than the largest value attributable to external concentration gradients of glucose around the cell.

These results contradict the suggestion of Rothstein et al. ('51) that glucose is transported across the yeast cell membrane by an enzyme like carrier the activity of which is supposedly depressed by uranium adsorbed to the cell surface. On the contrary, they indicate that (1) the first enzymatic reaction undergone by glucose occurs in a region internal to the cell surface (2) the uranyl inhibition is produced via a depression in the membrane permeability to glucose (3) the rate of entry of glucose is proportional to the difference in external and internal concentration over an external concentration range of at least 0 to 200 mg %. The relevance of the Langmuir and Schaefer effect is discussed.

CONCLUSIONS

1. Glucose penetrates the yeast cell by diffusing through a surface barrier which is impenetrable to UO_2 and then reacts with an enzyme (probably hexokinase) in some interior region of the cell.

2. The inhibitory effect of the uranyl ion on the glucose utilization of intact yeast cells is produced entirely by the decrease in membrane permeability caused by this ion.

3. The evidence at present does not support the suggestion that glucose entry into yeast is accomplished via an enzymatic carrier mechanism.

4. The yeast cell membrane permeability to glucose is essentially constant over a concentration range of 0–200 mg %.

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THE CAPACITY OF THE KIDNEY OF THE MARINE DOGFISH, *SQUALUS ACANTHIAS*, TO SECRETE HYDROGEN ION^{1,2}

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ONE FIGURE

INTRODUCTION

The remarkably constant urinary pH of the marine dogfish, *Squalus acanthias*, has long been of interest to renal physiologists (Pitts, '34; Smith, '39a; Hodler, Heinemann, Fishman and Smith, '55). The urine of these fish has been shown by these previous investigators to remain within the range of pH 5.6 to 5.9 in spite of the administration of Diamox, disodium phosphate (Na_2HPO_4), sodium bicarbonate, ammonium chloride, and maleic acid. Hodler et al. ('55) reported that any increase in hydrogen ion (H^+) excretion in this pH range is accompanied by a similar increase in phosphate excretion. Parenteral administration of Na_2HPO_4 further increased the titratable acid excretion, but the maximal rate of phosphate secretion by the dogfish tubule seemed to limit this increase.

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The lack of change in pH after Diamox has been attributed by these latter authors to the absence of carbonic anhydrase in the dogfish kidney. Another characteristic of the acid secretory mechanism in this organism is that the urine is acidified high in the proximal tubule (Kempton, '43) in contrast to the isohydric proximal tubular urine of amphibia (Montgomery and Pierce, '36). Thus, Kempton observed during direct micropuncture studies of the dogfish tubule that the proximal tubular urine was acid as shown by the direct injection of phenol red into the tubular lumen. Further, a saturated solution of disodium phosphate was promptly acidified when injected into the proximal tubular lumen (Kempton, '58).

The present study was undertaken to determine whether some change in the dogfish urinary pH could be induced by the administration of the weak base trimethylamine-HCl (TMA-HCl). In a previous communication from this laboratory (Cohen, Krupp, Chidsey and Biltz, '59) it was reported that TMA-HCl is excreted in the urine by net renal tubular secretion. We concluded that the amine appears in the urine primarily by the diffusion of the unionized free base from alkaline plasma into the acid urine where it is "trapped" as the trimethylammonium ion. With such high rates of excretion which are not limited, as is PO_4 , by a maximal rate of transport, one would expect either a rise in urinary pH or a marked stimulation of H^+ production by the kidney to maintain the fixed urine pH.

Indeed, we have observed that the dogfish urinary pH can be raised by TMA; in addition, experiments are reported which demonstrate that the dogfish kidney has the capacity to increase its rate of H^+ production several fold in spite of the absence of carbonic anhydrase in this organ.

METHODS

The collection and care of the dogfish and the experimental techniques used were exactly as described previously (Cohen,

Krupp, Chidsey and Biltz, '59). Inulin was determined in urine and plasma by the acid resorcinol technique of Schreiner ('50). The volatile amines in urine and plasma were determined by microdiffusion in the presence of formaldehyde (Conway, '50). As noted in a previous communication (Cohen, Krupp and Chidsey, '58), the endogenous volatile amine in dogfish urine has not been identified and, therefore, will be referred to as the Free Amine. In the experiments in which TMA was administered, the volatile amine excretion is also referred to as Free Amine since the microdiffusion technique as used does not permit differentiation of the amines. However, after TMA injection the Free Amine is undoubtedly TMA.

It should be noted that in the experiments in which multiple injections of TMA-HCl were given, the plasma concentration of Free Amine was not constant. Thus, large variations in excretion of Free Amine were observed in contrast to the constant excretion rate during the subcutaneous infusion of TMA-HCl.

Titrateable acid (T.A.) was determined by titration of the urine to pH 7.45 with 0.01 N NaOH using a Beckman pH meter. Urinary pH was determined immediately after removal from the collection balloon without anerobic precautions since it has been shown that the CO₂ content of dogfish urine is negligible, even after large injections of sodium bicarbonate (Hodler, Heinemann, Fishman and Smith, '55). Inorganic phosphate was determined in trichloroacetic acid filtrates of urine by the method of Gomori ('42).

RESULTS

In table 1 are shown the results of a representative experiment which was performed to determine the characteristics of the renal excretion of TMA while the plasma level of the amine was maintained constant. This was done by administering the TMA in a continuous subcutaneous infusion to a dogfish swimming in an aerated sea water tank (Cohen, Krupp, Chidsey and Biltz, '59).

During the last three observation periods, when the plasma amine concentration was maintained relatively constant, the rate of amine excretion exceeded its rate of filtration several fold. This leaves no doubt that the amine reaches the urine by net tubular secretion. The ratios of Free Amine U/P: Inulin U/P during these last three periods range between 15 to 19. Since W. W. Smith ('39b) has shown that the filtration fraction of the dogfish is 0.05 or less, these ratios indicate

TABLE 1

An experiment showing the net renal tubular secretion of amine during the continuous subcutaneous infusion of trimethylamine hydrochloride

1.8 Kgm Dogfish

TIME in HOURS	GFR	URINE FLOW	FREE AMINE					INULIN U/P	FREE AMINE U/P / INULIN U/P
	ml/hour	PLASMA	URINE	U/P	FILTERED	EXCRETED			
		μMol/ml			μMol/hr				
-16 to 0	Not Done	1.9	0	3	—	—	6	—	—
+1	Start Infusion TMA-HCl 0.5 at 24 ml/hr (0.67mMol/Kgm/hr)								
0-2.4	11.3	3.7	0.5	6.9	14	6	26	3.6	4
2.4-5.6	9.3	3.9	0.8	68	85	8	267	2.4	37
5.6-9.4	8.0	3.6	2.3	94	41	18	338	2.2	19
9.4-13.5	8.5	3.8	3.0	104	35	26	395	2.3	15
13.5-23.6	7.9	3.3	2.3	91	40	18	300	2.4	17

that virtually all the amine brought to the kidney is excreted into the urine during a single passage through the renal vascular bed.

The appearance of such large quantities of base in the urine suggested that a change in urinary pH might have occurred. That this, in fact, did occur may be seen in figure 1. The experiment shown is one of 4 similar ones in which repeated injections of TMA-HCl were given to a dogfish as indicated at the bottom of the figure. In each experiment there was a definite rise in urinary pH from control levels. The highest

Dogfish 38-7 5.6 kgm ♂

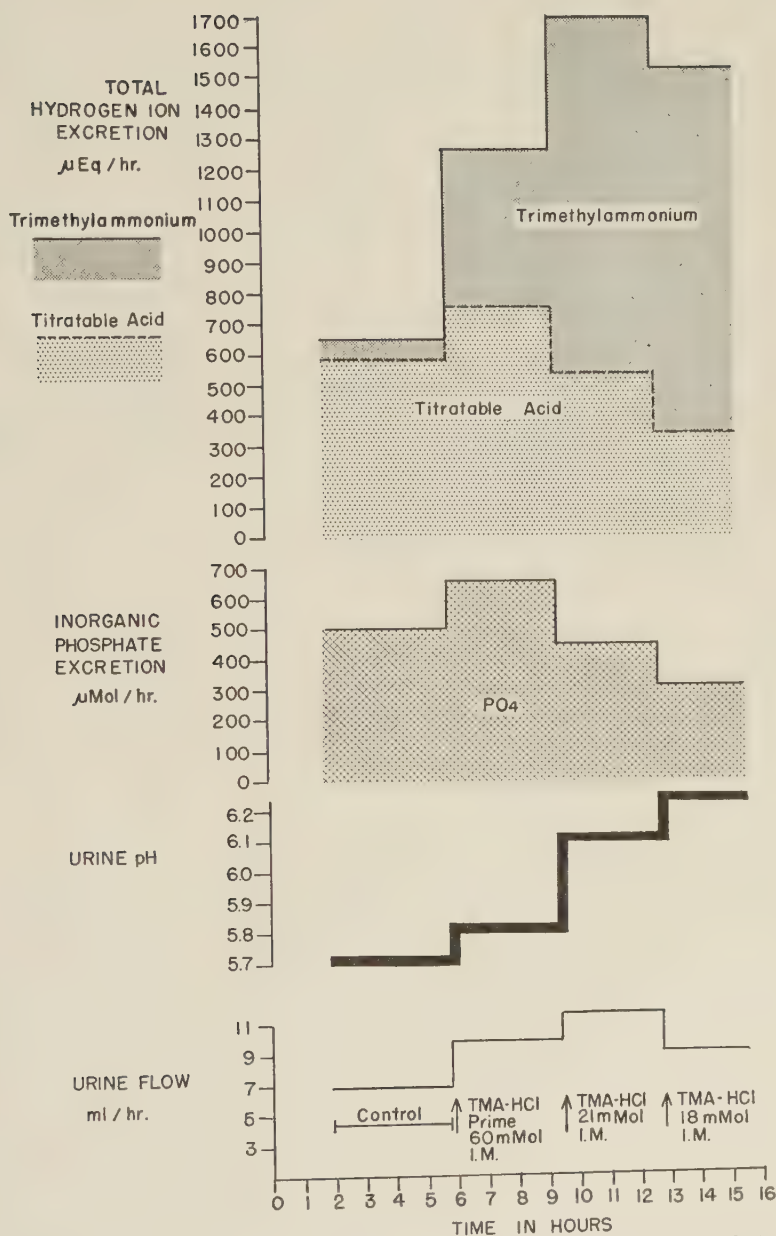


Fig. 1 The changes in urinary pH, titratable acid, inorganic phosphate and amine excretion during repeated injections of TMA-HCl.

urinary pH observed was 6.31. Such decreases in H^+ concentration, though small, have never been observed previously.

In this experiment it should be noted that the titratable acid (T.A.) excretion rate increases only transiently and then falls after the amine is given. There is a parallel change in the rate of inorganic phosphate (PO_4) excretion. However, in spite of the rise in urinary pH, T.A. excretion is equal to or exceeds slightly PO_4 excretion. This was observed in three of the 4 experiments and will be discussed below.

Assuming that all the amine excreted reaches the urine as the free base and is buffered there by the secreted H^+ , the total H^+ excretion is then approximated by the sum of T.A. and trimethylammonium. In figure 1, the total H^+ excretion has thus been increased about three fold above the control level. In another experiment with a 6.8 kg dogfish, the sum of T.A. and trimethylammonium rose from 200 $\mu Eq/hr.$ in the control observation to 2400 $\mu Eq/hr.$ after TMA, the urinary pH rising from 5.83 to 6.31.

Such large H^+ excretion rates are far in excess of those reported by Hodler et al. ('55) taking into consideration the differences in the size of the subjects used in the two studies. Further, this increased acid secretion reveals the capacity of the dogfish tubule to secrete large amounts of acid if the pH gradient between cell and tubular lumen is not great.

DISCUSSION

Whether the net tubular secretion of TMA occurs by an active transport mechanism or by the attainment of diffusion equilibrium for the free base between alkaline plasma and acid urine cannot be determined from these data. However, the rise in urinary pH indicates that the amine must reach the urine as the free base. The fact that the urine first becomes acid high in the proximal tubule and remains acid until excreted indicates that the blood perfusing the entire peritubular capillary network is continually exposed to a steep pH gradient. This reduces to a minimum the limitations

imposed on diffusion by time and surface area of exposure. The observed concentration gradients for TMA between urine and plasma in table 1 are also consistent with attainment of diffusion equilibrium. These facts indicate that TMA excretion most probably occurs by facilitated diffusion.

The large H^+ excretion rates reported here are far in excess of those observed by Hodler et al. ('55) in spite of the larger size of the subjects used in this study. In their report, maximal T.A. excretion rates of 100 $\mu\text{Eq/hr.}$ were observed with dogfish weighing between 1.5 and 3.0 kg. In figure 1 above, after TMA administration there was a maximal H^+ excretion of 1700 $\mu\text{Eq/hr.}$ in a subject weighing 5.6 kg. Thus the relatively great capacity of the dogfish kidney to secrete acid is revealed in these experiments.

A possible explanation for the large total H^+ secretion observed in this study as compared to the quantities reported by Hodler et al. ('55) may be found in the differences in the mechanism of renal excretion for TMA and PO_4 .

One must assume that the fixed urinary pH in the dogfish is due to a limiting electro-chemical gradient against which the tubular cell is capable of secreting H^+ . This limit must be approximately 1.7 pH units. When large amounts of PO_4 are secreted into the urine, increased H^+ secretion is possible until this maximal pH gradient is attained. Thus, the maximal rate of H^+ secretion would depend upon the rate of PO_4 secretion. This is analogous to the observations of Pitts and Alexander ('45) in the dog in which the magnitude of T.A. excretion was shown to be dependent upon the quantity and type of buffer present in the tubular urine. However, in the experiments with TMA large quantities of free amine diffuse into the acid tubular urine and are trapped there as the trimethylammonium ion. As with the increased active tubular secretion of PO_4 , further secretion of H^+ is "permitted" due to the decrease in pH gradient between tubular cell and urine.

That the rate of diffusion of the amine into the urine must have exceeded the maximal rate of H^+ secretion is seen by

the rise in urinary pH. Although the total H^+ secretion was much increased, this was inadequate to maintain a low urinary pH. The progressive rise in pH indicates that the limit of this H^+ secretory mechanism was being approached. This capacity of the dogfish kidney to increase the rate of H^+ secretion cannot be related to the enzymatic hydration of CO_2 since carbonic anhydrase is not present in the dogfish kidney. Thus other sources of the H^+ must be considered (Davies, '57).

The almost exact equality of the rates of excretion of PO_4 and T.A. in the face of a rising urinary pH was not expected. These T.A. and PO_4 excretory rates are within the same range of values reported by Hodler et al. ('55) if corrected for differences in weight of the fish. One would have expected that the T.A. should decrease relative to the PO_4 excretion as the pH rose during high amine excretory rates. That this did not occur indicates that small quantities of some unidentified buffers besides PO_4 are present in the urine of fish receiving TMA.

SUMMARY

1. Trimethylamine has been shown to be excreted by the dogfish kidney by net tubular secretion. At the plasma levels studied, the amine excretion is limited by the renal blood flow.
2. This large renal excretion of trimethylamine is associated with a rise in urinary pH which has never been observed previously in the dogfish.
3. The sum of titratable acid and trimethylammonium excretion reveals the relatively large capacity of the dogfish kidney to produce hydrogen ions in spite of the absence of carbonic anhydrase from this organ.
4. The maintained equality of the rates of inorganic phosphate and titratable acid excretions in spite of a rise in pH indicates that small quantities of other buffers are present in dogfish urine under the conditions of these experiments.

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A STUDY OF VASCULAR CHANGES IN SKIN GRAFTS IN MICE AND THEIR RELATIONSHIP TO HOMOGRAFT BREAKDOWN ¹

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NINE FIGURES ⁴

INTRODUCTION

Considerable interest has been shown in the cause and the mechanism of the rejection of skin grafted from one individual to another. There is some evidence that the reaction has genetic aspects (Loeb et al., '43). The response is more than a foreign body reaction in that the graft becomes, for a time, physiologically united with the host. The delayed reaction suggests in many ways an allergic response by the host (Gibson and Medawar, '43; Medawar, '44), and many aspects of the response are highly suggestive of an antigen-antibody reaction, including the fact that the reaction is a systemic rather than a local one (Medawar, '44). The latter possibility is not ruled out by reported failures to identify or isolate a specific antibody by standard immunological procedures.

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Because of the enigmatic character of the response, it seemed worthwhile to examine more closely the actual events as they occur both in the normal course of graft establishment and rejection, and in cases where this normal course could be modified by special treatment. Because the vascular changes are most obvious and could reasonably be expected to be an important factor in the graft breakdown, this aspect of the homograft problem is the subject of the present report.

Skin was chosen for study because it could be transplanted orthotopically into a position where it could be easily accessible for repeated examination. In this way it became unnecessary to rely solely upon interpretation of fixed specimens to reconstruct the changes in circulation of a graft, since these could be observed directly in the living tissue.

An important factor in any study of homo or heterotransplantation is the determination of the survival time of the transplant. Several methods have been used. The one selected for this study was that devised by Taylor and Lehrfeld ('53) in which, by daily microscopic examination of the living intact graft, the time after grafting at which stasis took place could be recorded. With this method, the vascular condition of the graft could be determined without subjecting the transplant to unnatural or damaging conditions, and repeated observations on the same graft could be made.

MATERIALS AND METHODS

The mice used in this study were all young adult males and females of two strains: albino and C3H. The rats were all young adult females from a colony of Wistar albinos maintained at this laboratory.

Grafts

All grafts were made on skin in the center of the dorsum from which the hair had been plucked 20 days before to insure that all transplants would be in the resting stage of the hair cycle.

On the trunk of the mouse and rat the skin proper, with its muscle, is separated from the fascia of the deep muscles by a subcutaneous layer of very loose connective tissue. All blood supply to the skin of the mid dorsal region reaches it through vessels which leave the underlying fascia in the axillary or inguinal region and course through the skin parallel to its surface and just beneath the panniculus carnosus. Numerous secondary vessels run in the plane just superficial to the panniculus carnosus in the deeper portion of the dermis.

Skin for grafting was obtained and sites for grafting prepared by careful dissection along the junction of dermis and panniculus carnosus. This type of preparation, referred to as a "dissected-type" graft, left intact the highly vascular panniculus muscle to serve as a graft bed. In contrast, if the entire skin is removed from the underlying fascia, as in the "full-thickness" type of preparation, a bed is left that is devoid of vascular supply. The proximity to a rich vascular supply in the dissected type graft guaranteed that restoration of graft circulation would take place more completely and more rapidly than in the full-thickness type.

Postoperative observations were made daily with the animal under light anesthesia, at which time fresh dressings were applied. The observational technique used was that described by Taylor and Lehrfeld ('53), the graft being examined *in situ* on the living animal under magnification which allowed visualization of the red blood cells within the capillaries.

In addition to this superficial examination on the living intact animal, the grafts were studied by means of three other techniques. Some animals were sacrificed at various stages in the life of the graft and the grafts prepared for histological study.

Other animals were injected *in vivo* with bromophenyl blue and the amount of dye entering the grafts observed. The technique was done to verify the microscopic examination of blood flow. The dye solution was made up after the method

described by Scothorne and McGregor ('53). With the animal under anesthesia, 0.05 ml of this solution was injected into the external jugular vein. As this dye is non-toxic, injections could be made in the same animal on successive days.

The third technique involved the injection of the vascular system with an india ink suspension. Via cardiac puncture, the vascular system was first washed out with citrated saline followed by injection with an india ink suspension. Care was taken to avoid too great a pressure in the solution which might rupture blood vessel walls. The graft was then removed and fixed. One half was cleared in oil of wintergreen for whole mount study, and the other half was prepared for histological study.

Daily observations, bromophenyl blue injections and histological examinations were made in the following animals: 33 autografts on white mice; 46 homografts between white and C3H mice; 53 homografts between white mice; 17 heterografts on white mice from white rats.

Daily observations and india ink injections were made on the following animals: 15 autografts on white mice; 20 homografts between white mice; 6 heterografts on white mice from white rats.

Modification of the time of stasis

In order to ascertain whether modifying the length of the survival time of the graft might also alter the vascular changes occurring at breakdown, two procedures were used.

In one procedure, 10 second-set homografts (grafts applied 10 to 12 days after first-set grafts that had been taken from the same donor) were applied to albino mice. Such second-set homografts have been shown to survive for a shorter time than the first-set grafts, (Medawar, '44, '45). The grafts were examined daily and biopsies taken at periodic intervals for histological study.

In the second procedure, 10 animals bearing homografts were subjected to cortisone therapy in order to prolong the life of the graft. A subdermal injection consisting of 0.5 ml

of an aqueous solution containing 12.5 mg cortisone acetate ⁵ was administered every three days starting on the day of the operation. These were also examined daily and biopsies were taken for histological study.

Disruption of vascularization to established grafts

In order to determine whether the rejection reaction could be simulated in established grafts by depriving them of blood circulation, the edges of 14 auto- and homografts (5 days post operative) were recut deeply enough to sever all vessels of the skin and underlying graft bed. This could effectively eliminate blood flow because the main supplying vessels run parallel to the surface.

Disruption of vascularization to normal skin

As a further attempt to show whether degeneration of homografts could be due entirely to stasis, the effect of a lack of blood supply to normal skin was determined. Two techniques were used to prevent blood from circulating through an area of normal skin. Sixteen albino mice were used.

The first method involved isolating a graft-sized area of skin by cutting its margins to full-thickness depth. As a result all the blood vessels to the area were severed leaving it entirely without blood circulation.

The second technique involved the destruction of the vessels to a graft-sized area by freezing around it with solid carbon dioxide. An area slightly larger than graft size was outlined on the skin and a knife-edged piece of solid carbon dioxide was held in contact with this outline for two to three minutes. If no blood flow was seen in the area in the following 48 hours, the destruction of all vascular connections to that area was considered complete.

In both cases the areas deprived of vascular supply were observed closely in the succeeding hours and the following

⁵ Supplied as "Cortogen" through the courtesy of Schering Corp., Bloomfield, N. J.

day. Biopsies for histological examination and india ink injections were made at periodic intervals.

OBSERVATIONS

Autografts

Six, 20, and 24 hours postoperative. In general, the sequence of vascular changes visible in autografts studied in the intact mouse paralleled those found in the rat (Taylor and Lehrfeld, '53; Woodruff and Simpson, '55), but the timing was slightly different.

No blood vessels could be seen in the grafts when they were examined *in situ* 6 hours after the operation. Likewise in histological preparations of grafts fixed at this time even the larger blood vessels could be detected only with difficulty since most vessels were collapsed and rarely contained any blood. Two factors could conceivably be responsible for this condition. First, since the skin was put under stretch in the process of dissecting, it was subjected to a distortion which could express much of the blood from the tissue. Second, as postulated by Hynes, ('54), it is probable that, as the graft was cut, the vessels went into spasm expelling their contents. After this first spasm the vessels lost their tonus, as a result of anoxia and the lack of sympathetic innervation, and were thus converted into flacid, empty channels but still capable of accepting any fluid drained into them.

Aside from this condition of the blood vessels, the other tissues of the graft itself appeared normal. In the host tissues which form the bed of the graft, all vessels were normally filled with blood. Here, in some cases, there had already appeared a mild white cell invasion.

By surface examination after 20 hours a few relatively large vessels containing stagnant blood could often be seen in the graft. Since this blood must have come from the host, it seems probable that, in these cases, at least one capillary sprout from the host skin had bridged the space between host and graft tissue and connected with an old graft vessel.

Through such a minute connection blood could seep into and gradually fill up some of the empty channels in the graft without detectable flow through these vessels.

In histological preparations of samples fixed at this time some vessels were still collapsed, others appeared to be dilated with fluid while a few were engorged with red blood cells. At this time the leucocytic infiltration of the graft bed was much greater than at 6 hours and was beginning to invade the graft.

By the end of the first 24 hours postoperative, surface observation showed many vessels to be filled with blood. This blood, though appearing to be static, was still fluid and without clots, as demonstrated by its displacement with the application of local pressure. The caliber of the filled vessels was usually slightly greater than that of comparable vessels in normal intact skin.

Although minute vascular connections between graft and host had obviously become established by this time, no blood flow through the graft vessels was detectable, even by microscopic examination. This must have been due to the extremely small caliber of the few existing connections in relation to the size of the vascular pool in the graft. The extremely slow rate of exchange through these connections was emphasized by the failure of bromophenyl blue to enter the graft and by the fact that no ink was seen in the graft after systemic injection of the host, even though all vessels of the host were filled with ink (fig. 1). In these cases large vessels of the bed extending upward towards the graft were distended with ink up to the line of incision. While the vessels within the graft were free of ink, they did contain blood cells.

Histological observations showed that the graft epidermis was still normal whereas the dermis showed some edema and most of its larger vessels were dilated and completely filled with red blood cells. Leucocytic infiltration was seen, not only in the bed but also in the graft itself.

Forty-eight hours postoperative. By the end of the second day, the picture, as seen by superficial observation, was much the same as the day before. The pattern of blood-filled ves-

sels now appeared to be quite complete. In the majority of grafts, however, there was still no detectable blood flow and in most dye-injected animals no bromophenyl blue entered the graft. India ink, after injection, likewise generally failed to appear in the graft.

At this stage, however, exceptions were encountered in which sluggish flow could be seen in a few of the graft vessels. Also after ink injection a few large vessels sometimes contained ink and some small ink-filled channels could be found which connected the filled vessels of the graft with arteries or veins of the bed. The increase in the number and size of such connections was directly correlated with the appearance of circulation and the rate of blood flow through the graft vessels.

Histological study of the graft revealed that the epidermis was still thin, as in the resting stage of the hair cycle, and the white cell infiltration had progressed almost up to the level of the epidermis (fig. 2). The dilated graft vessels still contained many cells but some of these vessels were not as greatly engorged as at the first day.

Seventy-two hours postoperative. By 72 hours following transplantation steady blood flow was generally in evidence throughout the graft. Both bromophenyl blue and ink injection verified the presence of good vascular function. It seemed evident that this circulation was via the old vessels of the graft and not by way of new vessels formed after transplantation. The time interval between grafting and appearance of blood flow was too short to allow for the formation of new vessels of the size observed. In addition, there was no detectable change in the *pattern* of vessels from the time when they were first filled with blood cells until circulation was completely restored. Furthermore, during the initial period when circulation was lacking, there was no histological indication of degeneration of the graft vessels. No observations were made to determine whether these old blood vessels were eventually with new ones after the graft had become completely established.

Histologically the grafts were in much the same condition as they were at 48 hours after transplantation although now the vessels were not as densely packed with blood cells.

Ninety-six hours postoperative and later. In most grafts the rate of blood flow was normal by 96 hours postoperative and the vessel caliber was returning to normal. Many grafts were entirely normal in appearance, and on subsequent days the line of suture between the graft and the host skin became less and less distinct. After the 10th day hair growth usually became evident.

An apparently normal pattern of ink filled vessels was seen in the graft after injection with india ink, and it remained unchanged even after 10 days (fig. 3). The vessels, while more distended than those of normal skin, became progressively smaller as they neared the epidermis. By this time, vascular connections between host and graft were seen coming from the adjacent host into the graft as well as those coming up from the bed. Biopsies made on later days showed about the same picture, with the vessel caliber gradually approaching that found in normal skin.

From the 4th day on the histological picture was nearly normal, the only difference being that the subdermal connective tissue layer was thicker and contained more leucocytes and a greater proportion of cells were seen in the dermis. Gradually even these differences disappeared.

Homografts

Restoration of circulation. In all homografts the vascular changes and their timing were the same regardless of whether the grafts were from animals of the same strain and/or sex or from different ones.

During the 5 days immediately following the grafting, the sequence of vascular changes and their timing were identical with that described for autografts.

As in autografts, histological examination showed that by 48 hours postoperative the vessels were packed with cells.

This engorgement, however, decreased during the following 24 hours. The epidermis was normal, as was also the dermis excepting for a slight edema and leucocytic infiltration which appeared to be no more than an inflammatory reaction to the trauma of operation (fig. 4).

From the fourth day on to the time of stasis the grafts appeared normal except for the presence of a more cellular dermis. The latter also contained more extravascular white blood cells and a thicker layer of subdermal connective tissue.

While these observations are in agreement with those of Medawar ('44) and Scothorne and McGregor ('53), they vary from the findings of Ham ('52) and Conway et al. ('51a, '51b, '52). Conway by means of a modified Algire chamber studied revascularization of autografts and homografts and claimed that host blood vessels grew inward toward the periphery of the graft but failed to enter the graft tissues by 7 to 8 days. However, by their own admission, the opacity of the graft made it impossible to ascertain whether there was penetration of the graft by vessels from the underlying graft bed. Thus these may have become vascularized earlier from the bed and the failure of the vessels to enter the periphery of the homografts within 7 to 8 days would then be due to the fact that homograft breakdown had already begun. Furthermore it is quite likely that these grafts might have been adversely affected by their environment which was more abnormal and artificial in the chambers Conway employed than in the fitted surface defects used in this study.

Rejection. Although the onset of homograft rejection was most clearly and definitely identified by stasis in the graft, this might be preceded as much as 24 hours by a distention of the vessels. The average time for the appearance of the homograft rejection reaction, using the more striking character of hemal stasis as the indicator, was between 7 and 8 days after implantation of the graft. The extremes of variation were from the sixth to the eleventh day.

Hemal stasis, while becoming complete within a relatively short period of time (24 to 48 hours), was nevertheless a grad-

ual process. Following the dilation of the vessels of the graft there was a decrease in the rate of blood flow. The vessels became engorged with cells and finally all blood flow stopped. In some cases this process occurred uniformly throughout the graft within 24 hours. In other instances, some vessels reacted earlier than others so that within the same graft part of the vessels contained static blood while in other vessels the blood was flowing slowly.

Ink injected at the time of breakdown entered only those vessels through which blood had still been flowing. If stasis was complete at the time of injection, no ink would enter the graft (fig. 5).

Histologically there was no detectable morphological change in the graft vessels prior to stasis. Neither was there any clotting of blood. After stasis, however, the contents of the vessels and their endothelial lining showed a progressive degeneration, the first sign being an hemolysis of erythrocytes and the presence of pyknotic leucocytes. This was followed by further degeneration of the white blood cells and pyknosis of the endothelial cell nuclei (fig. 6). Finally there was complete degeneration of the walls of the vessels leaving only fragmentary remnants to mark their former sites (fig. 7).

In cases where all the vessels immediately below the epidermis were in some stage of degeneration, the deeper dermal vessels were still either normal or only slightly degenerated, regardless of whether these deeper vessels were arterioles, venules or capillaries (fig. 8).

Thus these findings seem to indicate that the cessation of blood flow was a gradual process starting in the regions immediately below the epidermis and progressing toward the bed, and that the degenerative changes followed this stasis.

No pathological changes in the graft tissue were noted until after stasis was complete. However, once circulation in the graft had stopped, degenerative changes in the graft epidermis and dermis occurred rapidly. By 24 to 48 hours after complete stasis the epidermal elements were dead, as evidenced by the homogeneity of the cytoplasm, loss of distinct

cell boundaries and failure of the nuclei to stain, and the dermal cells and fibers were in some stage of degeneration. Paralleling the observations on blood vessels, dermal degeneration also progressed from the epidermal surface downward into the graft, being further advanced in the superficial regions than in the deeper dermis.

Modifications of the rejection reaction

It was hoped that something more might be learned of the nature, and possibly the cause, of the rejection reaction if the latter could be modified so as to alter the intensity or the timing of the events which characterized it. Three methods were used in this attempt: heterografting, second-set homografting and homografting with cortisone therapy.

Heterografts. The heterografts between mice and rats were performed with the thought that the greater genetic difference between donor and recipient might increase the degree of violence of the graft rejection reaction and thus possibly make visible some morphological changes not seen during the milder process occurring in homografts. However, with the exception of their timing, the events seen in these grafts were essentially the same as those in homografts. The return of circulation and the incorporation of the heterografts took place exactly as in homografts and autografts. In most cases the rejection reaction occurred at 6 days after grafting although it ranged between 5 and 7 days. This is one to two days less than the survival time of homografts. By 8 days the graft was sloughing. The vascular changes were identical to those described for homograft breakdown, in both their gross and histological aspects.

Second-set homografts. It has been repeatedly shown that second-set grafts have a shorter life than first-set grafts from the same donor, (Gibson and Medawar, '43), (Medawar, '44, '45), (Billingham et al., '52), (Lehrfeld et al., '54). In the case of the second-set homografts applied to mice in this study, the course of events was the same as that taking place in first-set

homografts except that breakdown commenced three to 4 days sooner. The reaction was the same as that seen in first-set grafts and was no more violent. Histologically there was no difference between the two sets of grafts.

This is contrary to the findings of Medawar, ('44, '45), and of Billingham et al. ('54b), who state that in second-set grafts the reaction between graft and host is limited to the graft bed and no more leucocytes invade the graft than do so during graft establishment. They report also that original restoration of circulation is never complete. It was found, in this study, that in the rejection reaction infiltration by leucocytes did occur in the transplant and, while it might not be quite as widespread throughout the graft, it nevertheless was present to a greater extent than that seen as a result of the initial traumatic inflammation.

Homografts with cortisone therapy. It is known that cortisone acetate therapy will increase the survival time of homografts although eventually they too will break down, (Billingham et al., '51a, '51b; Sparrow, '53). In this experiment the homografts in mice which received injections of cortisone were maintained for 15 to 18 days. After this time the breakdown of the grafts took place in the usual manner. From surface examination and histological studies nothing could be detected in the process which differed from that of untreated homografts.

These results indicated that shortening or extending the survival time of homografts did not appear to alter in any way the nature of vascular changes in breakdown.

Disruption of vascularization to established grafts

In order to determine whether the degenerative changes in the homograft could be accounted for by the lack of blood supply only, the vascular connections to well-healed autografts, as well as to homografts prior to the time of breakdown, were severed.

The results in the following 48 hours were of two types. In the first type, although blood flow had ceased, the vessels were not distended by 24 hours following the incising, but by 48 hours the graft dermis was showing definite signs of degeneration. In the second and more usual type by 24 hours following severing, the vessels were moderately to highly distended, producing a typical reaction picture.

There was no detectable difference between the events occurring after stasis in a rejected homograft and those found after severing the vascular connections of a well-established homograft, nor between those events occurring in an incised autograft compared with a rejected homograft. The typical pathological changes seen in homograft breakdown, such as leucocytic and fibroblastic infiltration of the dermis, progressive degeneration of the blood vessels and their contents, and finally progressive degeneration of the epidermis and dermis were all found to occur in these grafts.

Disruption of vascularization to normal skin

The first method involved cutting to full-thickness depth around a square area of the skin of the same size as a graft. In all cases it was found that by the first 24 hours after cutting, only a few large vessels were visible at the periphery and these contained static blood, but were only slightly distended. By 48 hours few, if any, vessels were visible and no flow was evident.

By 24 hours the epidermis was normal but the stroma beneath the panniculus carnosus seemed more fibrous than normal. There was a white blood cell infiltration here which extended outward into the dermis. The deep veins of the adjacent skin were packed with cells. The vessels of the isolated area contained hemolyzed red blood cells and pyknotic leucocytes but the endothelium was still intact. The dermis showed varying degrees of degeneration. By 72 hours the epidermis was degenerated but still attached. Some collagen fibers were degenerating and all the dermal cells were dead.

Leucocytic infiltration was present throughout. The endothelium of the superficial vessels was gone and that of the deeper vessels was degenerating.

The second technique involved freezing the margins of a graft-sized area of skin with solid carbon dioxide to a full-thickness depth. The effects upon the unfrozen central area were observed from the surface in the succeeding hours and the following day. From one-half to 5½ hours after freezing, no blood vessels were visible. Twenty-four hours after freezing the area was grayish white in color and few if any vessels were visible. In subsequent days the area dried and sloughed, reacting much the same as a homograft.

Examination of histological preparations showed that after 5 hours there was some leucocytic infiltration in the deeper portions of the dermis. The blood vessels were still completely normal but becoming engorged with cells. By 24 hours the epidermis showed slight degeneration but the glandular and dermal elements were normal. Moderate white blood cell infiltration was present throughout the dermis. The blood vessels were packed with cells. The superficial vessels had normal endothelium in most cases although a few showed degeneration in varying degrees (fig. 9). All contained partially hemolyzed cells. The deeper vessels were normal and the cellular content was, for the most part, healthy.

India ink injections were done both for comparison with those done on normal grafts as well as to make certain that all blood supply to the area had actually been interrupted. The results were the same as those seen in homo- and heterografts after "rejection" had taken place. After 5 days, the ink-filled vessels were seen only in cases where the injury had been insufficient to prevent vascular connections from reforming. In those cases which showed no blood flow in the area, there was no ink penetration.

These tests showed that the degenerative changes which follow experimentally induced stasis in normal skin and autologous grafts parallel closely those seen in homograft and heterograft rejection. By 48 hours the area deprived of cir-

culatation closely resembled that of a homograft 72 hours after rejection stasis. The apparent acceleration of the degenerative changes in this case was probably due to the fact that the cessation of blood supply was sudden and complete throughout the graft, while it was gradual in the case of homograft breakdown.

DISCUSSION

The events occurring during homograft breakdown were characteristic of an acute inflammatory reaction, and more specifically of the Arthus reaction. Gerlach ('23), on the basis of a complete microscopic study of the Arthus phenomenon, concluded that the reactions were in no way specific and did not differ from any other inflammatory reaction, except in degree.

There are many indications in the literature that the Arthus phenomenon results from an interference with the blood circulation. Fröhlich ('14) concluded that the entire reaction was due to an allergic phenomenon.

The prominent feature of the histopathology of the Arthus reaction is vascular damage and it seems likely that the blood vessels are sensitized. Abell and Schenck ('38) studied the reactions of blood vessels in the ear of rabbits sensitized to horse serum after the introduction of antigen. They observed the following: (a) arteriolar contraction, with the obliteration of the lumina of arterioles and vascular stasis; (b) increased adherence of leucocytes to the blood vascular endothelium and white blood cell emigration through the walls of the capillaries and venules; and (c) sticking of leucocytes to each other to form clumps or emboli which blocked the circulation. Repeated introduction of serum caused further leucocytic emigration, leucocytic clumping, erythrocytic extravasation, and finally endothelial destruction.

Rich and Follis ('40) found that the Arthus reaction could be induced only in vascularized tissue and concluded that the phenomenon was not due to the sensitization of tissue, but rather to the sensitization of the capillaries.

Thus, many of the observations of this study seem to fit the description of events occurring in the Arthus reaction. However, in the latter, the blood vessels have been reported to become filled with thrombi resulting in stasis. The rejection of homografts has also been reported by some workers to be associated with thrombosis of the vascular bed (Conway et al., '52). However, Barnard ('55) pointed out that these agglomerations of erythrocytes were not actually thrombi as they were devoid of a fibrinoid matrix. Knisely et al., ('47) also reported that in a great many diseases and pathologies the blood cells agglutinated into masses which caused a thickening of the blood and produced a circulating sludge. If this condition progressed far enough it resulted in weakened, dilated, leaking capillaries and venules and eventual stasis produced by blockage of the vessels with masses of agglutinated red blood cells.

This is in accord with the results of this study where it was found that just prior to and at stasis the vessels, although engorged with cells, contained no clots. This engorgement of the vessels may be a factor responsible for the slowing and final cessation of flow. The engorgement, in turn, is probably the result of one of two factors or a combination of both. First, it may be due to a hemoconcentration produced by increased capillary permeability which is characteristic of an inflammatory reaction. The edema noted at this time gives support to this concept. Second, it is possible that a pseudoagglutinin is either locally produced or pre-existent in the circulation and locally released. Barnard ('55) postulates that just prior to stasis a change occurs in the graft which may produce one of the following situations:

1. it may render the intracapillary red blood cells more susceptible to pseudoagglutination;
2. it may release or produce a pseudoagglutinin intravascularly; or
3. a pseudoagglutinin may be elaborated extravascularly and diffuse into the vessels and effect erythrocytic clumping.

The change which produces or releases this pseudoagglutinin is probably brought about in some way by the interaction of an antibody whose nature is as yet uncertain, with an antigen in the graft.

Following hemal stasis, the graft progressively degenerated until no cellular elements remained. Billingham et al. ('54a) claimed that one to three days may pass before the beginnings of overt tissue breakdown are visible and three to 4 days until this process is complete. They felt that a delay of this kind could not be merely the result of lack of blood supply to the tissue. On the other hand, Scothorne and McGregor ('53) found epidermal destruction far advanced within 24 hours after vascular failure and felt that this rapid breakdown argued against the concept that graft destruction was due to vascular stasis. Conway et al. ('53a), using the chamber technique in the study of mouse skin homografts, attributed the graft destruction to nutritional lack. On the other hand, Medawar maintained that the blood transports a destructive substance to the graft. If this is true it would seem that a graft could survive only if it does not become revascularized. This was claimed by Merwin and Hill ('54) and Algire et al. ('54) who found that homografts of thyroid and Harderian gland were destroyed only if they became vascularized. Surviving nonvascularized grafts were presumably supported by diffusion of nutrients and metabolites. However, if these grafts become vascularized, they were then destroyed. It must be remembered that such grafts, to survive without revascularization, must be very small, usually not more than 1 mm square. If the dosage concept of Medawar is correct, it is probable that grafts of such size are too small to elicit a rejection response except when transplanted to an already immunized host.

Grafts of the size used in this study die as quickly from a failure to reestablish circulation initially as they do from secondary vascular stasis.

The results of this study strongly indicate that this tissue degeneration, like the Arthus phenomenon, is a direct result

of vascular failure. The degeneration of the graft tissue follows the degeneration of the vessels starting from the epidermal-dermal junction and progressing into the deeper portions of the dermis to the graft bed. In addition it was found that the same results were produced after destroying the vascular connections to either healthy grafts or normal intact skin. The only difference was the slightly more rapid rate of degeneration after induced vascular failure as compared with that produced as a result of homograft vascular stasis. It seems probable that this can be explained by the fact that in the former case vascular failure was sudden and took place throughout the area while in the latter case vascular stasis occurred progressively over a longer period of time.

CONCLUSIONS

1. The rate and extent of the restoration of circulation in dissected-type auto-, homo-, and heterografts of mouse and rat skin have been studied by means of direct microscopic examination; intravenous injections of a diffusible vital dye, bromophenyl blue; india ink injections of the vascular pattern; and histological study.

2. Restoration of blood flow took place at the same rate and to the same extent in all three graft types, the average time being 48 to 72 hours postoperative.

3. The sequence of events occurring in the graft during establishment of circulation are as follows: (a) expelling of blood elements from the vessels; (b) dilation of the vessels; (c) engorgement of the vessels by erythrocytes entering from a few minute capillaries which have grown from the bed and connected with the old host vessels; and (d) blood flow.

4. It is the old graft vessels which function in this early circulation rather than newly formed vessels.

5. Vascular stasis is one of the first visible signs of the onset of homo- or heterograft rejection and all other morphological changes follow vascular failure.

6. Stasis occurs 7 to 8 days postoperative in homografts and 5 to 7 days postoperative in heterografts.

7. Neither increasing nor decreasing the survival time of the graft modified the nature or violence of the rejection reaction.

8. Stasis occurs earliest at the epidermal-dermal junction and progresses downward through the deeper portions of the graft.

9. The vascular and circulatory changes following stasis are characteristic of an acute inflammatory reaction of the Arthus type.

10. Degeneration of the graft elements followed this vascular degeneration downward through the graft from the epidermal surface.

11. It was concluded that the degeneration of the graft following stasis is a direct result of vascular failure.

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Abbreviations to plates

B, graft bed CAP, capillary EP, epidermis G, graft H, host

PLATE 1

EXPLANATION OF FIGURES

All figures are photomicrographs. Sections are $6\ \mu$ thick.

- 1 Whole mount at the junction of a 24-hour p.o. autograft and its host skin after india ink injection. Note that the functional blood vessels are confined to the bed of the graft. $\times 50$.
- 2 Section of a 48-hour p.o. autograft showing moderate leucocytic infiltration throughout the dermis. $\times 525$.
- 3 Whole mount of a 10 day p.o. autograft after india ink injection. $\times 56$.
- 4 Section of a 72-hour p.o. homograft showing leucocytic infiltration throughout the dermis. $\times 112$.
- 5 Whole mount of a 6-day p.o. homograft after india ink injection showing the ink in the bed vessels only (after stasis). $\times 52$.

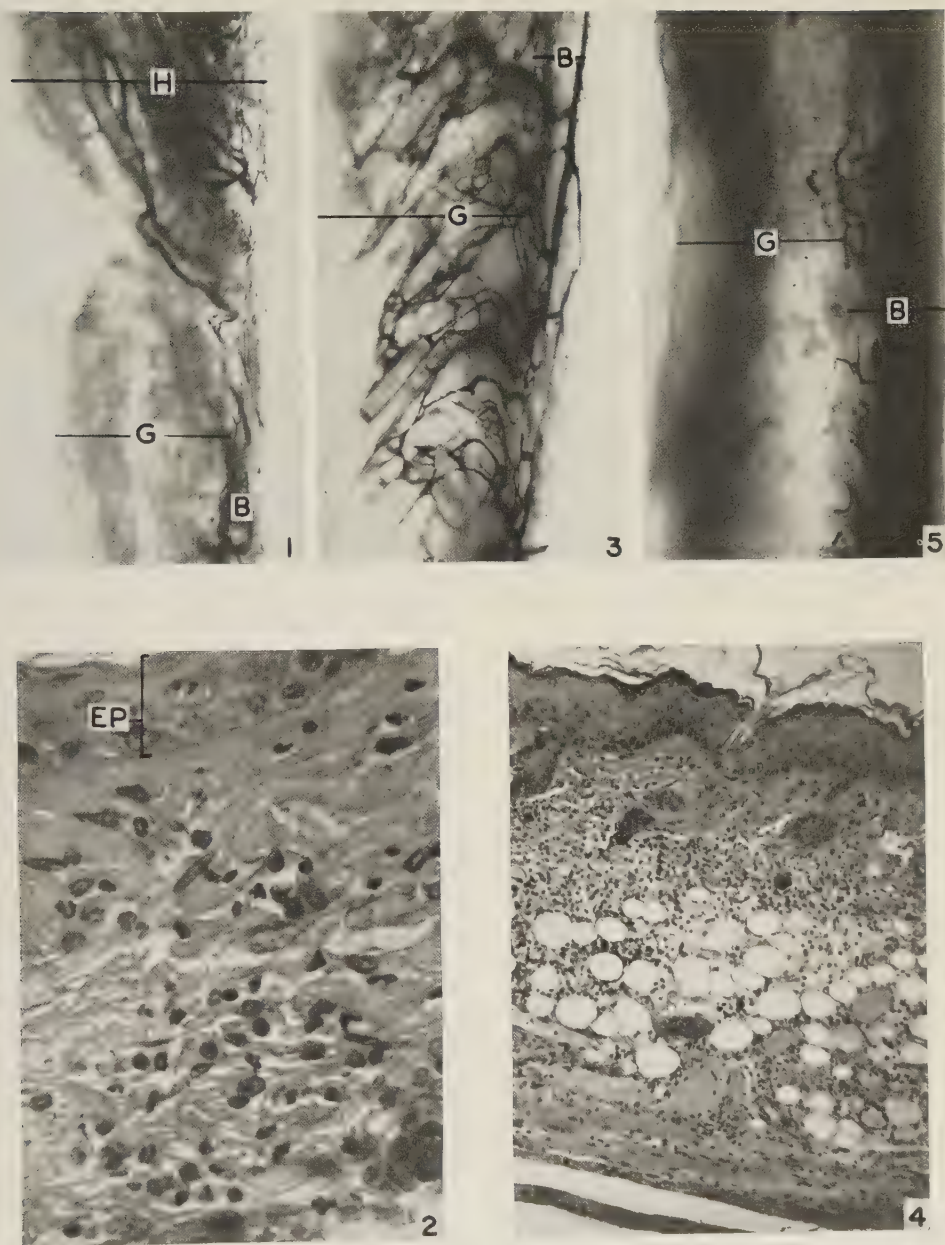
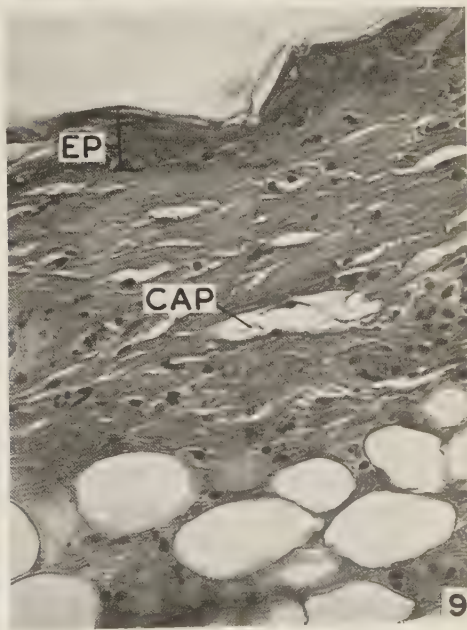
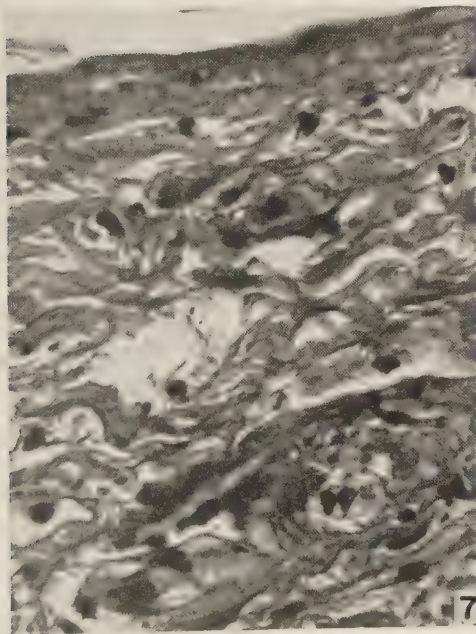
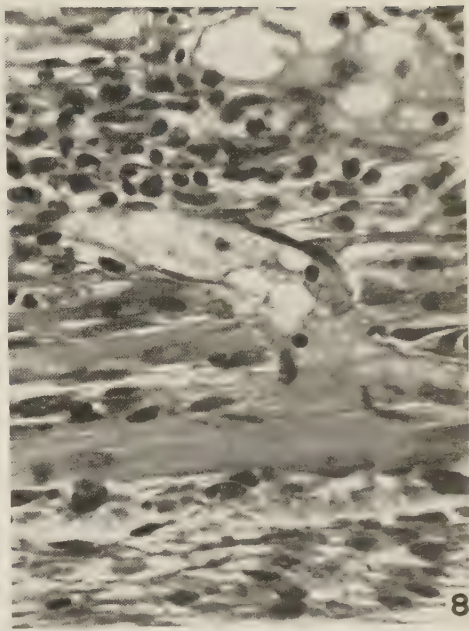
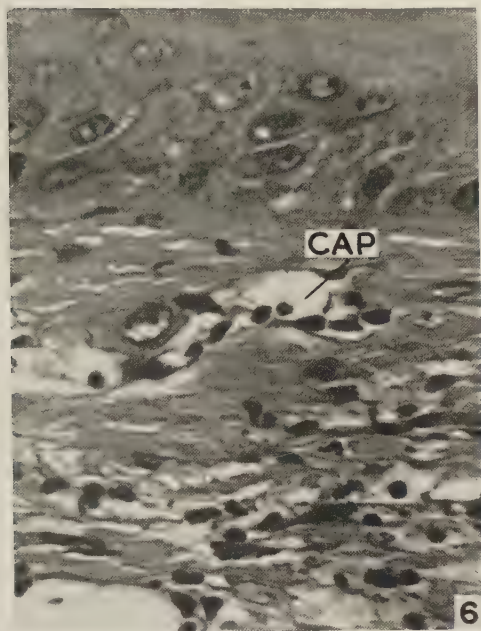


PLATE 2

EXPLANATION OF FIGURES

All figures are photomicrographs. Sections are $6\ \mu$ thick.

- 6 Section of an 8-day p.o. homograft showing a degenerated capillary just beneath the epidermis. This graft, at the time of biopsy, showed stasis in the superficial vessels. $\times 560$.
- 7 Section of a 9-day p.o. homograft showing the degenerated epidermal and dermal cells and the lack of recognizable blood vessels. $\times 560$.
- 8 Section of the same 8-day p.o. homograft as shown in figure 6, showing a deep dermal capillary containing some hemolyzed and some normal erythrocytes and slightly pyknotic leucocytes. $\times 560$.
- 9 Section of normal skin 24 hours after its vascular connections had been disrupted by freezing the margins of the area with solid carbon dioxide. Note the partially degenerated blood vessels, the few pyknotic dermal cells and the degenerated epidermal cells. $\times 240$.



TRANSMEMBRANE FLUX OF K^{42} IN *ACANTHAMOEBA* SP.

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FOUR FIGURES

INTRODUCTION

The present study is a preliminary report on the nature of ion fluxes in a representative of the Phylum Protozoa, namely, *Acanthamoeba* sp. (Neff, '57). It was undertaken in view of the sparsity in the literature of permeability studies of any sort involving protozoans, and as a continuation of earlier osmotic studies on isolated *Acanthamoeba* mitochondria (Klein and Neff, '59). It is hoped that this paper will be of comparative value with ion flux studies in single cell systems such as yeast (Conway and Downy, '50), bacteria (Cowie, Roberts and Roberts, '49), Ascites tumor cells (Maizels, Remington and Truscoe, '58) and others.

The data presented includes the following: water, K and Na contents of vegetative cells, cysts and mitochondria determined by various methods; occluded space in centrifuged cells; transmembrane fluxes in vegetative cells and cysts using K^{42} , with a few experiments using Na^{24} ; and the effect of temperature, K concentration and added calcium on these fluxes and the net loss of K.

METHODS

Cell growth

Amoebae were grown in specially adapted two liter culture bottles containing 600–800 ml of constantly aerated liquid

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medium with the following composition per liter; 1% proteose peptone (Difco Certified), 1% dextrose, 120 mg NaCl, 272 mg KH_2PO_4 , 3 mg CaCl_2 anhydrous, 3 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 3 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. The pH was adjusted to 5.5. Non-aerated (stationary) cultures were maintained in 500-ml Erlenmeyer flasks for use as inoculum. One flask containing 40–50 ml of a 7–21-day-old stationary culture was used to inoculate each culture bottle. Approximately 10 ml of centrifuged amoebae cells were harvested from one aerated bottle after 7–8 days growth at 28–30°C. It was found earlier that amoebae in aerated cultures would entirely encyst if left for 11–14 days.

Mitochondrial preparation

Cells were washed twice in 0.308 M sucrose and centrifuged at $600 \times g$ for 5 minutes or less. A glass homogenizer was used to disrupt the cells and the debris-nuclear fraction was eliminated by centrifuging at $800 \times g$ for 5 minutes in a Sorvall angle head centrifuge. The sucrose supernatant containing mitochondria and microsomes was then centrifuged at $10,000 \times g$ for 35 minutes at 2–5°C in an International refrigerated centrifuge with a high speed head. Mitochondrial pellets were checked microscopically for purity and resuspended in appropriate solutions for ion analyses and water content determinations.

Water content

The water content of vegetative cells, cysts and mitochondria was determined gravimetrically using a chainomatic balance. Preparations were dried in porcelain or platinum crucibles at 115°C for two to three hours in a hot-air oven. Volumes of cells, cysts and mitochondria were determined in calibrated centrifuge tubes and in modified hematocrit tubes.

Cation analyses

Na and K analyses were determined at 589 and 767 m μ , respectively, using a Beckman flame spectrophotometer. Quan-

titative recovery of cations in solution from the several preparations was accomplished by ashing for 24 hours at 650°C, homogenization, or boiling in 5 N HNO₃ for 15 minutes. All preparations were finally brought up to volume and contained 5% TCA; any precipitate was centrifuged out.

Occluded space

Occluded or extracellular space in cells centrifuged for 5–10 minutes at 600–800 × g was determined by two methods. A sucrose method was used in which a known volume of packed cells was suspended in 0.308 M sucrose to a given volume. The cells were centrifuged out and the dilution of the sucrose in the supernatant was determined by dry weights. Similarly, centrifuged cells occluding 0.308 M sucrose were suspended in distilled water and then centrifuged out. The gain in dry weight of the supernatant was measured.

In the second method the change in percent transmission of a hemoglobin solution at 540 mμ was determined in a Beckman DU spectrophotometer. A known volume of cells diluted a standard hemoglobin solution due to occluded space. The increase in transmitted light through the hemoglobin solution was then a measure of the dilution caused by the occluded space.

Isotopes

K⁴² and Na²⁴ were obtained from Oak Ridge National Laboratories dissolved in HCl. They were subsequently neutralized with NaOH and appropriately diluted for use. Counts were made in a constant geometry counting rack using an end window type Geiger tube and scaling circuit. Corrections were made for counting time, half-lives and self absorption. Total counts were arranged to yield a standard error of less than $\pm 2\%$. The exchange of isotopes was determined by following the disappearance of counts from a standard radioactive suspending medium and the appearance of counts in measured cell samples. The procedure involved periodic sampling from a homogeneous suspension of cells and centrifugation of these

samples for 5 minutes at $800 \times g$. Known volumes were then pipetted from the separated supernatants and cell pellets (resuspended in distilled water) and placed in planchettes to be dried before counting. The medium used for flux studies contained; 0.02 M KH_2PO_4 or NaH_2PO_4 , 0.005 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05 M dextrose and 0.104 M NaCl or KCl. When the concentration of either K or Na was varied, the concentration of the other cation was adjusted to maintain a total of 0.104 M. The osmolarity of the medium is approximately 0.154. This medium is referred to as "ASM", amoeba suspending medium.

RESULTS

Description of Acanthamoeba sp.

This small soil amoeba can be grown in pure clonal culture, in large numbers and in ordinary liquid media (Neff, '57). The cells have an average diameter of approximately 20μ , and 20–40% of their volume is occupied by mitochondria (Klein and Neff, '59). The latter are mostly spherical and measure $0.5\text{--}1.0 \mu$ in diameter. Under appropriate conditions amoeba cysts will form; they have an average diameter of approximately 14μ . The walls of the cysts thickened and become irregular with age. For photographs and a more complete description see Neff ('57).

Occluded space

Occluded space in centrifuged *Acanthamoeba* cell pellets was found to be 17.4, 21.0 and 14.5% in three sucrose experiments and 19.7, 14.6, 15.0 and 13.0% in 4 hemoglobin experiments. An average value of 17.0% was chosen to be used as a correction factor in calculations of water and cation contents, and in flux studies.

Cations and water content

Table 1 contains a compilation of data including water, Na and K content of vegetative cells, cysts and mitochondria.

Preparations were washed two times in approximately 50 ml of either sucrose, ASM, NaCl, KCl or distilled water before analyses. To release intracellular cations, vegetative cells were either ashed, homogenized or boiled in HNO₃; cysts and mitochondria were boiled in HNO₃. Comparable results were obtained from the various methods. Nitric acid boiling usually gave slightly higher values.

TABLE 1

Water, Na and K contents of Acanthamoeba vegetative cells, cysts and mitochondria prepared in various wash media

NO. OBS.	WASH MEDIUM	PERCENT WATER	NA	K	AVERAGE K:NA
mM/l H ₂ O					
Vegetative cells					
28	0.308 M sucrose	80.4 ± 2.4	32.5 ± 12.7	56.5 ± 12.8	1.77
20	0.154 M ASM	80.7 ± 1.0	30.5 ± 9.0	60.0 ± 11.9	
24	0.154 M NaCl or KCl	79.7 ± 2.6	33.1 ± 11.2	56.7 ± 15.7	
4	Dist. water	—	14.3 ± 3.6	26.9 ± 1.7	
Cysts					
10	0.308 M sucrose	89.6 ± 0.6	9.3 ± 1.0	5.2 ± 0.6	0.58
8	0.154 M ASM	91.3 ± 1.5	10.9 ± 2.1	5.8 ± 2.1	
4	0.154 M NaCl or KCl	90.0 ± 1.0	9.5 ± 2.8	6.6 ± 1.3	
4	Dist. water	—	6.8 ± 1.2	4.0 ± 0.4	
Mitochondria					
6	0.308 M sucrose	69.2 ± 6.0	28.9 ± 6.0	25.1 ± 4.0	0.87

The average water contents were approximately 80.4% for vegetative cells, 90.3% for cysts and 60.2% for mitochondria by weight. The average Na concentrations were approximately 32.4 mM for vegetative cells, 9.9 mM for cysts and 28.9 mM for mitochondria per liter of cell or particle water. The average K concentrations were approximately 57.3 mM for vegetative cells, 5.7 mM for cysts and 25.1 mM for mitochondria per liter of cell or particle water. The apparent low values of Na and K in vegetative cells washed in distilled water were also accompanied by an increase in cell volume of 1.8 to 2.0 times that in isotonic medium. Cyst volume was not affected by distilled water washing.

In calculating the water content of cysts, the correction factor of 17% occluded space was used. Although one would expect a larger occluded space in packed cysts due to their relatively rigid walls, it can be calculated that more than 60% of packed cyst volume would have to be extracellular in order to lower the cyst water content of 90.3% to the vegetative cell level of 80.4%. The relative percentage of young or old cysts (i.e., rounded or irregular walled) gave no difference in results.

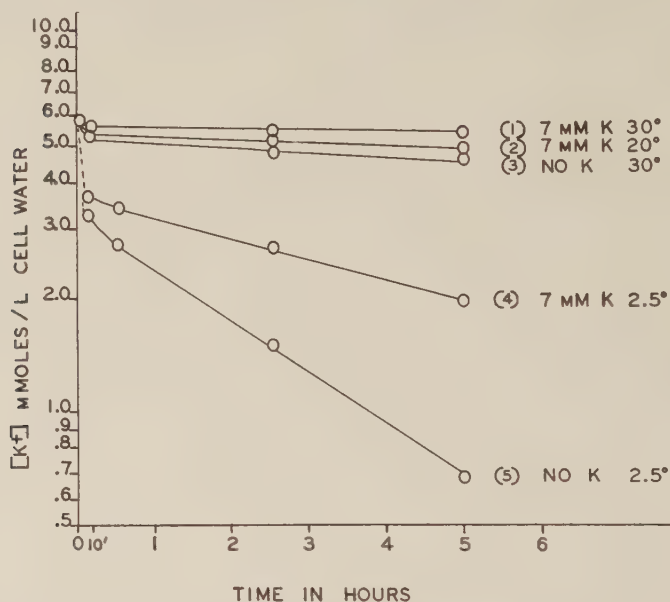


Fig. 1 A semi-log plot of net K loss from *Acanthamoeba* with time at different temperatures and extracellular K concentrations.

The values for water, Na and K in isolated mitochondria have been taken from a limited number of determinations and, therefore, should be considered approximations at most.

*Effects of temperature and K concentration
on net K loss*

In figure 1, mMoles of K per liter of cell water is plotted on a semi-log scale versus time in hours. Each curve repre-

sents the average of at least 2-6 experiments. If the amoebae are suspended in media with varying amounts of K at several temperatures, they lose K. A study of this phenomenon was performed as a preliminary to exchange experiments in order to establish the conditions in which a steady state prevailed. The net loss curves are characterized by an initial rapid component followed by a slower single exponential one. The rapid phase occurs in less than 5-10 minutes. The net loss of K at 30°C with 7.0 mM and 0.2-0.7 mM K in the ASM is shown in curves (1) and (3), respectively. When very little K is present (curve 3) approximately 9% of the cell K is lost initially at a rate of 50-150 mM per hour. After the initial

TABLE 2

Q₁₀'s for net K loss from Acanthamoeba at two extra cellular K concentrations

CELLS	ASM	K _i :K _o RATIO	AVERAGE Q ₁₀ 'S	
			2.5-30°C	20-30°C
	mM K/l			
57.3	0.2-0.7	127:1	1.33	—
57.3	7.0	8:1	4.36	3.26

phase and during the following 5 hours, the rate of loss is reduced to approximately 1.5 mM per hour. When 7.0 mM K is present (curve 1) 5-6% of the cell K is lost initially at a rate of 30-100 mM per hour. However, the loss during the slow component is less than 0.28 mM per hour. One may consider that after the initial phase the intracellular K content is relatively stable under the latter conditions. In a few experiments Na loss was found to be similar in a Na-free ASM at 30°C.

By comparing the slopes of the terminal component of the curves in figure 1 and dividing by 10° units of temperature change, one can calculate Q₁₀'s for net K loss at the two extracellular K concentrations investigated (table 2). When the K_i:K_o ratio is very large (127:1), the Q₁₀ is low (1.33). When this ratio is reduced to 8:1, the Q₁₀ is increased to 4.36 over the temperature range between 2.5-30°C. With 7.0 mM K present the Q₁₀ between 20-30°C is 3.26.

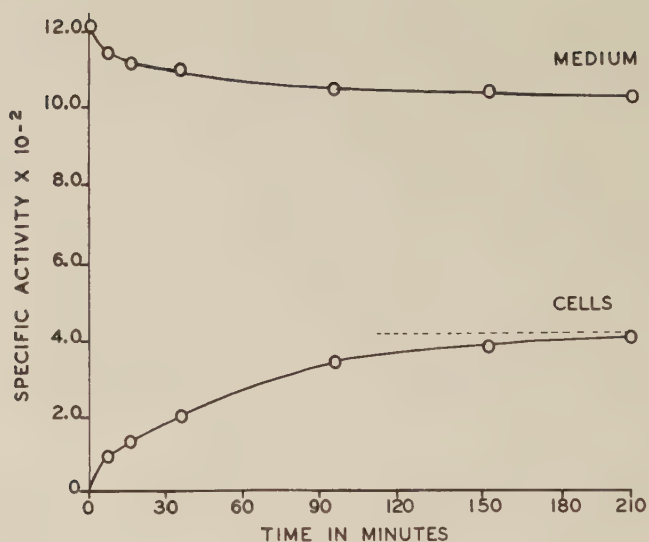


Fig. 2a K^{42} exchange in *Acanthamoeba* with 6.75 mM K in medium at 30°C. Cells contained 64.6 mM K/l cell water.

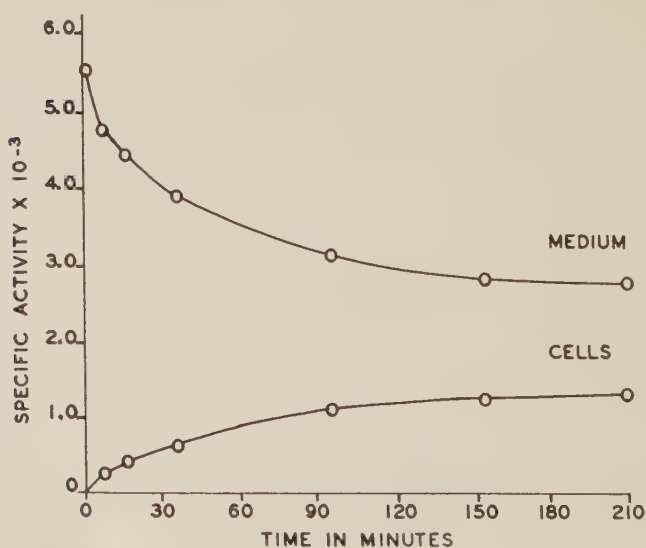


Fig. 2b K^{42} exchange with less than 1.0 mM K in medium at 30°C. Cells contained 63.6 mM K/l cell water.

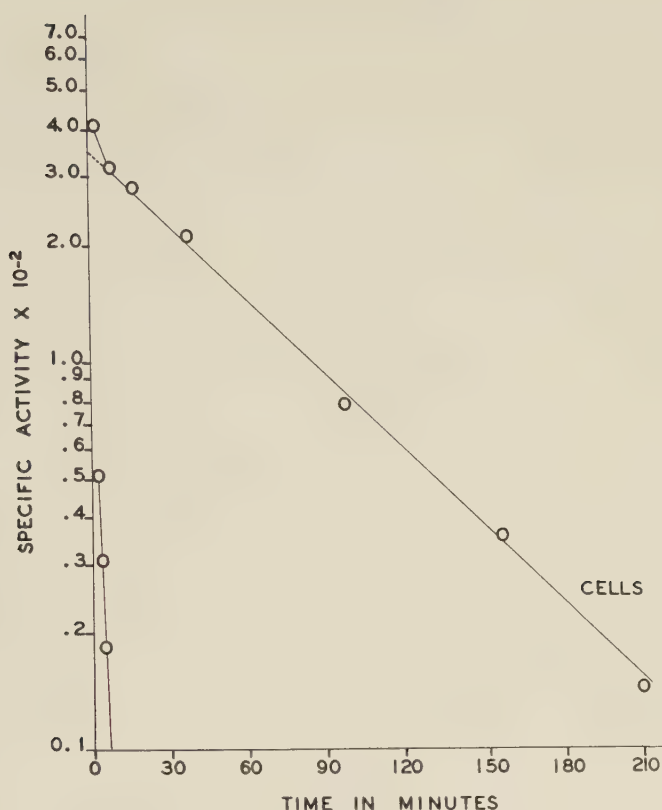


Fig. 2c A semi-log plot of net change of SA with time from cells-curve (fig. 2a). Two components are resolved.

K⁴² exchange in Acanthamoeba

Amoeba K exchange has been studied by following the appearance of K⁴² in cells and the disappearance of K⁴² from a standard radioactive medium. In figure 2 there are illustrated typical exchange experiments using two different K concentrations in the ASM. Specific activities (counts/min./eq K) for the medium and cell samples have been plotted versus time. In order to analyse the data, the general transport equation, formally identical to the one used by Solomon ('49), has been employed.

$$\frac{d [K^{42}]_{\text{amoebae}}}{dt} = K_1 [K^{42}]_{\text{medium}} - k_2 [K^{42}]_{\text{amoebae}} \quad (1)$$

In equation (1), k_1 and k_2 are the transfer coefficients for influx and efflux, respectively. The change of $[K^{42}]$ amoebae with time equals the influx $k_1 [K^{42}]$ medium minus the efflux $k_2 [K^{42}]$ amoebae. If one divides the equation by $[K]$ amoebae, which is essentially constant during the experiment, one obtains the following:

$$\frac{dSA \text{ amoebae}}{dt} = k_1 \frac{[K^{42}] \text{ medium}}{[K] \text{ amoebae}} - k_2 SA \text{ amoebae}, \quad (2)$$

where

$$SA \text{ amoebae} = \frac{\text{counts of } K^{42}/\text{min.}/\text{ml cell water}}{\text{meq. K/liter cell water}} = \text{counts/min.}/\text{eq K}$$

$$K^{42} \text{ medium} = \text{counts/min.}/\text{ml medium}$$

$$K \text{ amoebae} = \text{mM K/liter cell water}$$

$$k_1 \text{ and } k_2 = \text{transfer coefficients for influx and efflux, respectively, and are expressed as a number/unit time}$$

The slope of the curve $SA \text{ amoebae}$ is an estimate of $dSA \text{ amoebae}/dt$. At any experimental time t , $SA \text{ amoebae}$ $[K^{42}]$ medium and the slope as an estimate of $dSA \text{ amoebae}/dt$ are known. If one substitutes such values at two different times, t_1 and t_2 , into equation (2), one obtains two equations with two unknowns (k_1 and k_2) in each. The latter may be solved algebraically. In this case several combinations of slopes at points along the curves were determined during the terminal phase of net K change (see fig. 1), when the cells are in a steady state. The mean values are taken as the best estimate of the true value of the transfer coefficients. Corrections for all dilutions of supernatants and cells were made including carryover of counts in occluded space of centrifuged cell pellets. The average values obtained for k_1 and k_2 (fig. 2a) were $8.19 \times 10^{-4}/\text{sec.}$ and $2.20 \times 10^{-4}/\text{sec.}$, respectively. These transfer coefficients are assumed to be identical with those in equation (3). This involves the assumptions that the biological activity and distribution of K^{42} and K^{39} in the system are the same.

$$\frac{d [K] \text{ amoebae}}{dt} = k_1 [K] \text{ medium} - k_2 [K] \text{ amoebae} \quad (3)$$

The rate of influx into the amoebae equals $k_1 \times [K] \text{ medium}$ and the rate of efflux equals $k_2 \times [K] \text{ amoebae}$. The ex-

changeability of amoebae K can be expressed by the following relation:

$$\begin{aligned}\text{Exchangeability} &= \frac{k_1 \times [\text{K}]_{\text{medium}}}{k_2 \times [\text{K}]_{\text{amoebae}}} = \frac{(8.19 \times 10^{-4}/\text{sec.}) (6.75 \text{ mM/l})}{(2.20 \times 10^{-4}/\text{sec.}) (64.6 \text{ mM/l})} \\ &= \frac{5.53 \times 10^{-3} \text{ mM/l/sec.}}{1.42 \times 10^{-2} \text{ mM/l/sec.}} = 0.39,\end{aligned}\quad (4)$$

or 39% of the amoebae K is exchangeable. The values in different experiments ranged from 33 to 50% with approximately 7.0 mM K present.

Calculated values of k_1 and k_2 can be checked by substituting into the integral form of the general transport equation.

$$[\text{K}^{42}]_{\text{amoebae}} = \frac{k_1}{k_2} \cdot [\text{K}^{42}]_{\text{medium}} (1 - e^{-k_2 t}), \quad (5)$$

where $[\text{K}^{42}]_{\text{amoebae}}$ is to be found. This calculated value comes out to be 23,830 counts/min./ml cell water. The observed value was 25,175 counts/min./ml (fig. 2a). These values agree within approximately 5%.

It is also possible to estimate the absolute transmembrane flux of K by knowing the average diameter of the amoeba cell (20 μ), and by employing the formulae of Keynes ('51).

$$k_1 = \text{MA/CiV} \quad \text{and} \quad k_2 = \text{MA/CoV}, \quad (6)$$

where M = moles/cm² sec.; A = surface area in cm²; V = volume in cm³; Ci and Co = concentration of cation inside and outside in moles/cm³, respectively; and k_1 and k_2 = the transfer coefficients calculated above. Substituting in the values one finds:

$$\begin{aligned}M_{\text{influx}} &= \frac{(6.75 \times 10^{-6} \text{ M/ml}) (8.19 \times 10^{-4}/\text{sec.}) (4.189 \times 10^{-9} \text{ cm}^3)}{(1.257 \times 10^{-5} \text{ cm}^2)} \\ &= 1.84 \times 10^{-12} \text{ moles/cm}^2 \text{ sec.}\end{aligned}$$

$$M_{\text{efflux}} = \frac{(6.46 \times 10^{-6}) (2.2 \times 10^{-4}) (4.189 \times 10^{-9})}{(1.257 \times 10^{-5})} = 4.74 \times 10^{-12} \text{ M/cm}^2 \text{ sec.}$$

One pica mole = 10^{-12} moles, therefore, the absolute influx is 1.84 pmoles cm⁻² sec.⁻¹ and the absolute efflux is 4.74 pmoles cm⁻² sec.⁻¹.

If one arbitrarily sets the equilibrium value for the SA amoebae curve (fig. 2a) at a SA = 405 counts/min./eg K, and

then subtracts the SA values at points along the curve from this value, the net changes in SA with time will be determined. A plot of these values on a semi-log scale is shown in figure 2c. This curve is characterized by an initial rapid component followed by a slower single exponential one. If the values for the extended portion of the slow component curve are subtracted from values of the initial fast component portion of the curve, one can resolve the fast component alone. The result is the very steep curve in the lower left of figure 2c. The half lives of the fast and slow components are approximately three minutes and 46 minutes, respectively. The fast component accounts for 10–15% of the total exchangeable K.

Figure 2b shows the K exchange when less than 1.0 mM K was present in the ASM. This is an average of three experiments. The curves are similar in shape to those in figure 2a. The exchangeability was calculated to be 49%. As the cells have not reached a steady state as far as net K exchange is concerned (fig. 1) under the conditions of these experiments, any calculated values of k_1 and k_2 , and consequently exchangeability, are at most approximations of the true values.

Calcium effects

Figure 3 illustrates the effect of added 0.01 M CaCl_2 on K^{42} exchange in an ASM containing 6.75 mM K. Specific activity of cells and medium are plotted versus time. This particular experiment is typical and was chosen because it was performed under identical conditions, at the same time and with equal quantities of cells from the same culture bottle as that experiment plotted in figure 2a. The curves have been analysed as previously described. The average influx coefficient $k_1 = 6.64 \times 10^{-4}/\text{sec.}$; the average efflux coefficient $k_2 = 1.50 \times 10^{-4}/\text{sec.}$ Therefore, the rate of influx and efflux are as follows:

$$\begin{aligned} \text{Influx} &= (6.64 \times 10^{-4}/\text{sec.}) \ 6.75 \times 10^{-3} \text{ M/l (K in medium)} \\ &= 4.48 \times 10^{-6} \text{ M/l/sec.} \end{aligned}$$

$$\begin{aligned} \text{Efflux} &= (1.50 \times 10^{-4}/\text{sec.}) \ 64.6 \times 10^{-3} \text{ M/l (K in cells)} \\ &= 9.69 \times 10^{-6} \text{ M/l/sec.} \end{aligned}$$

The exchangeable amoebae K is 46.4%. Comparing these flux rates with those found previously in a Ca-free ASM (fig. 2a), one finds that added calcium reduces the rate of influx 19% and the rate of efflux 32%. In addition, direct analyses of the total cell and medium K indicated that during the experiment the cells in Ca-free ASM had lost approximately 8.5% of their original K content, most in the first 5–10 minutes. However, an equal concentration of cells in the Ca supplemented ASM lost little, if any, K.

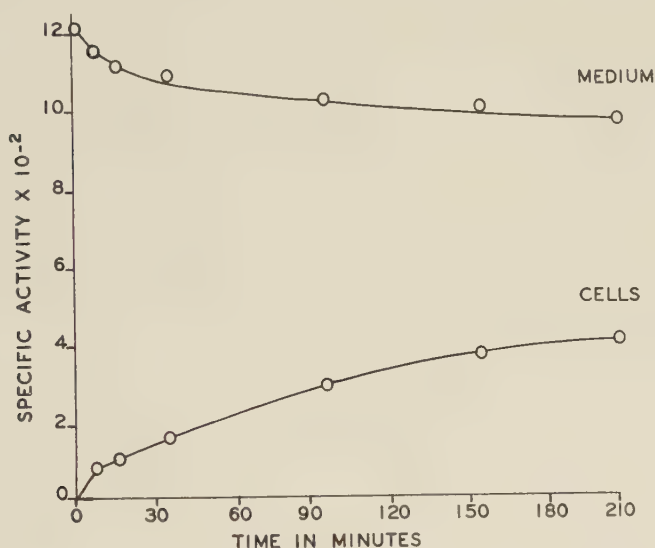


Fig. 3 K⁴² exchange in *Acanthamoeba* at 30°C when 0.01 M CaCl₂ is added to a medium containing 6.75 mM K. Cells contained 64.6 mM K/l cell water.

In other experiments, when no K was present in the ASM, 0.001 M CaCl₂ had little effect on K⁴² exchange or on the gradual net loss of intracellular K.

Na²⁴ fluxes

A few experiments were performed using Na²⁴. Specific activities of cells could not be calculated as only samples of the supernatant were taken. The same general type curves

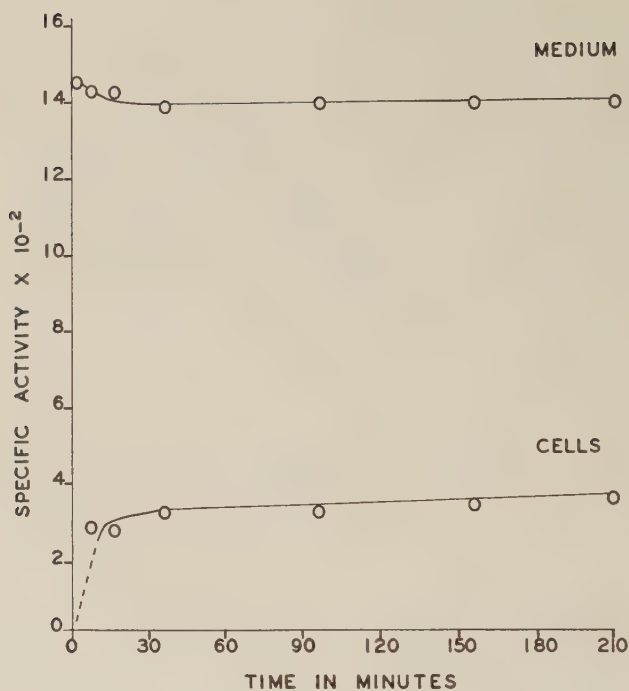


Fig. 4a K^{42} exchange in *Acanthamoeba* cysts with 7.0 mM K in the medium at 30°C. Cysts contained 5.9 mM K/l cyst water.

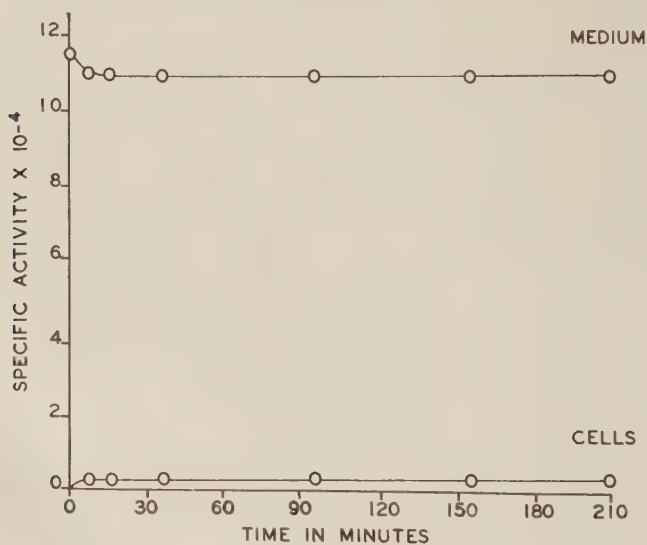


Fig. 4b K^{42} exchange in cysts with 0.1-0.4 mM K in the medium at 30°C. Cysts contained 5.8 mM K/l cyst water.

were found as with K⁴², that is, a fast component with a half-life of 5 minutes or less followed by a slower single exponential component with a half life of approximately 45–59 minutes.

K⁴² exchange in Acanthamoeba cysts

Figures 4a and 4b illustrate K⁴² exchange in amoeba cysts. Specific activities of medium and cysts have been plotted versus time so that the general transport equation might be applied. With 7.0 mM K in the ASM apparently only 20–25% of the cyst K is exchangeable (fig. 4a). Notice that most of the exchangeable fraction can be accounted for by the initial fast component. When very little K (0.1–0.4 mM) is present in the ASM, the exchangeability of cyst K is reduced to less than 5%, again almost all of which can be accounted for by the fast component (fig. 4b). Direct cation analyses show that cysts lose little, if any, K during 6 hours incubation in K-free ASM after the initial washing.

DISCUSSION

The occluded space in packed amoebae cells of 17% is comparable to the 14% in mouse Ascites tumor cells (Maizels, Remington and Truscoe, '58) which are of similar size, and the 23% in Baker's yeast (Conway and Downey, '50). The amoebae cell water content of 80.4% is also comparable with mouse Ascites tumor cells 86%, rabbit leukocytes 80% (Wilson and Manery, '49) and *E. coli* 75% (Cowie, Roberts, '49). The K:Na ratio in this amoeba is 57:32 mM. In mouse Ascites tumor cells it is 113:39 mM and in rabbit leukocytes it is 106:79 mM.

It is interesting to note that along with encystment, the amoeba cell loses approximately 90% of its K and 70% of its Na. The overall K:Na ratio reverses itself from 1.77 in the vegetative cells to 0.58 in cysts. This may be a reflection of a large decrease in respiratory machinery necessary for active cation uptake during encystment. Microscopic observation reveals a depletion of mitochondrial content. The increased

water content of the *Acanthamoeba* cyst and the visible depletion of mitochondria are apparently exceptions to the changes generally reported during encystment as stated by Van Wagendonk ('55), namely, "encystment is accompanied by a loss of water with a corresponding increase in the density of the protoplasm."

There is little difference in the Na concentration of whole cells and mitochondria, but the K falls to less than 50% in the latter, making the two cations approximately equal in concentration. If the mitochondria comprise approximately 33% of the dry weight of the amoeba cell (Klein and Neff, '59), the percentage of total cell K and Na in the particles can be calculated to be approximately 14 and 26%, respectively.

It was found that approximately 40% of the amoeba K was exchangeable. This amount was due to an initial rapid and then a much slower component of K flux. *E. coli* (Cowie, Roberts and Roberts, '49) and Baker's yeast (Conway and Downey, '50) show similar components. The fast components are comparable in time. In the yeast cell, the cell wall space, which is 10% of the total cell volume, was interpreted as accounting for the fast component. A plot of the data in a brief paper on the uptake of Na^{22} in *Pelomyxa carolinensis* (Nardone et al., '56) also reveals an initial rapid phase followed by a slower component before approaching an apparent equilibrium value. The fast component of cation loss and exchange in *Acanthamoeba* during washing and flux studies is believed to represent the fraction of cations loosely associated with the cell membrane. It is less likely that this 10–15% of the exchangeable K actually represents some component of the cytoplasm. One interpretation of the slower component of K exchange is that it represents the free K of the cytoplasm. The mitochondrial K may partially account for the relatively large non-exchangeable or very slowly exchangeable fraction. The remainder may be bound by lipid or protein. These amoebae appear to accumulate fat globules with age, possibly as a stored energy source. It would be interesting to determine the amount of cellular K associated with this fatty fraction.

When little K is present in the ASM, the net loss of K ions has a low Q_{10} suggesting predominantly a simple diffusion loss of cation due to a very steep concentration gradient. However, when 7.0 mM K is present, the loss of internal K has a relatively high Q_{10} now suggesting that an active maintenance process is unmasked which plays a more prominent role when a lesser $K_i: K_o$ concentration gradient exists. An increase in extracellular K may stimulate active K uptake. It appears to be well within the capability of the amoeba cell to maintain a concentration gradient of K inside to K outside of 8:1 (57 mM: 7 mM) in ASM. Actually, in the regular culture medium, actively growing cells maintain this gradient at least at 30:1. The apparent insensitivity to large reductions in extracellular K may be a reflection of the rather large slowly or non-exchangeable fraction of cell K borne out by the isotope studies.

If it is true that the initial fast component of exchange represents K loosely associated with the membrane, then the remaining cyst K is relatively non-exchangeable.

The fact that added Ca slows the rate of K efflux more than influx may be interpreted as due to an effect on membrane sites responsible for simple diffusion. This is strengthened by the finding that added Ca also inhibits spontaneous cation net loss down a concentration gradient. Conversely, Ca may inhibit the rate of K influx to a lesser degree as flux in this direction is primarily active.

SUMMARY

The water, Na and K contents of *Acanthamoeba* sp. vegetative cells, cysts and mitochondria have been determined. Transmembrane fluxes of K⁴² reveal an initial rapid and a terminal slower component, with approximately 40% K exchangeability in vegetative cells. Exchangeable K in cysts is considerably less. The effects of temperature and added calcium on fluxes and net cation loss are presented and discussed.

ACKNOWLEDGMENTS

The author is indebted to Dr. William C. Holland for his helpful criticisms and suggestions during the preparation of this paper.

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KINETICS OF LUMINESCENCE IN CHAETOPTERUS SLIME, AND THE INFLUENCE OF CERTAIN FACTORS THEREON ^{1,2}

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SEVEN FIGURES

Since the early work of Panceri (1878), luminescence of the polychaete annelid, *Chaetopterus*, has been the subject of occasional studies from various points of view, including histological as well as biochemical and physiological (cf. Harvey, '40, '52, '55, '57; Nicol, '52, '55; Sie, Chang and Johnson, '58³). Light emission is associated with two types of secretory phenomena. In one type, gland cells located chiefly in the aliform notopodia liberate into the surrounding medium, via the oral groove, a luminescent slime which under favorable conditions glows with a gradually diminishing intensity for a period of some minutes. In the other type, a flash extending over a period of some seconds occurs, chiefly in the segmented posterior notopodia, without the liberation of appreciable quantities of free luminescent slime, but accompanied by the local discharge of material from certain gland cells that contain granules and that apparently constitute the photogenic tissue.

¹ Contribution number 114 of the Hawaii Marine Laboratory.

² Aided in part by Contract Nonr 1353(00), Project NR 165-253, between the Office of Naval Research and Princeton University. Reproduction in whole or in part is permitted for any purpose of the United States Government.

³ The manuscript of the paper by Sie et al. was completed sooner, although the research was carried out later than that described in this paper.

As yet, no biochemical components of the light-emitting system have been isolated or identified, nor has a "luciferin-luciferase reaction" been demonstrated wherein light emission occurs on mixing, at room temperature, crude extracts of the photogenic tissue prepared in hot and cold water respectively.

The present investigation was undertaken in an effort to obtain further evidence on the nature and properties of the light-emitting system, primarily as it occurs in the free slime. Although attempts by various methods to enhance luminescence, or to restore it after decay, were either unsuccessful or of uncertain interpretation, data on the kinetics of decaying light intensity in various media, the influence of possible cofactors, and of inhibitors such as urethane and di-isopropyl-fluorophosphate (DFP) have revealed some hitherto undescribed characteristics of the system.

MATERIALS AND METHODS

The organisms studied were a variety of *Chaetopterus variopedatus*⁴ found in small parchment-like tubes, from about two to 8 inches in length, attached to, or winding through small recesses in, the coral of Kaneohe Bay near the Hawaii Marine Laboratory. In detaching the tubes it was often necessary to chip away the coral, bit by bit, with a hammer and chisel. Placed on a layer of sand in a tray of running sea water, the worms remained healthy inside their tubes for several days.

Luminescent slime was obtained in a dark room by first draining the sea water from the tube then pressing gently to irritate the worm. Individual specimens varied greatly in the amount and brilliance of the slime that could be obtained in this manner; some specimens, if they responded at all, would emit only a dim glow visible through the walls of the tube, whereas others would repeatedly liberate an essentially clear and colorless but brightly luminous material in sufficient

⁴Kindly identified by Dr. Olga Hartman.

amounts to drain several drops from the tube into a small vial in an ice bath. As a rule, two or three drops of the luminescent fluid were caught in 1 to 2 ml of sea water, or other medium as desired in the vial.

Within one to three minutes after collecting the slime, a 0.5-ml portion was introduced by means of a tuberculin syringe into a specimen tube in a water bath of an apparatus similar to that described by Johnson and Plough ('59). The temperature of the bath remained satisfactorily constant, without thermostatic control, during a given experiment. On different days it varied with room temperature through a range of about 23° to 26°C. The specimen tube was shielded from extraneous light and so arranged that it could be exposed, by opening a shutter, to an RCA type 1P21 photomultiplier connected through an amplifying system to an Esterline-Angus automatic recorder. Because of a wide variability in initial intensities and rates of decay of luminescence, not only in specimens from different worms but also from the same individual, no attempt was made to standardize accurately the elapsed time between collecting the slime and recording its luminescence, though recording was usually begun as quickly as possible and essentially always within three minutes or less.

During the course of decaying luminescence one or more solutions could be added through a three-way syringe stopcock without exposing the specimen to extraneous light or interrupting the continuity of the record. The volumes used were such that the total volume of luminescent fluid was always exposed to the window of the photomultiplier.

RESULTS

Order of decay and influence of the medium

Luminescence of the secreted slime in a small volume of sea water diminishes rapidly at room temperature (fig. 1), showing that one or more components necessary for light emission disappears. Aeration with air or oxygen scarcely

affected the course of decaying luminescence. Vigorous stirring usually had no effect, though on some occasions it caused a momentary increase in intensity, possibly by stimulating bits of photogenic tissue shed by chance with the slime. Addition of just sufficient sodium hydrosulphite ($\text{Na}_2\text{S}_2\text{O}_4$) to use up the dissolved oxygen stopped light emission, but aeration with air or with oxygen would then restore luminescence. Thus, while oxygen is required for luminescence, it is not the component whose disappearance is responsible for the progressive decrease of luminescence in these experiments.

Although during the initial period luminescence sometimes appeared to decrease in the manner of a first order reaction, the rate of decay generally decreased as time went on and luminescence occasionally persisted as a dim glow for many minutes. Figure 1 (A) illustrates a typical curve for a specimen of slime caught in about 9 volumes of sea water. A second specimen from the same worm was similarly caught in sea water concentrated by evaporation to contain double the normal salt content, yet the kinetics remained practically the same as before (fig. 1, B). No significant change in kinetics was observed when phosphate buffered NaCl solution ("PN," consisting of equal volumes of 0.5 M NaCl and 0.25 M $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$, pH 7.3) was used in place of sea water, nor was the course of decay appreciably altered by adding an equal volume of this solution during an early period (fig. 1, C). Similar results were obtained also with unbuffered 0.5 M NaCl (fig. 2, A). A freshly secreted drop of slime luminesced dimly even in 0.5 N HCl. A bright, brief flash of light occurred on addition of an equal volume of 0.5 N NaOH, and was followed by dim luminescence again. When fresh slime was added to several volumes of saturated $(\text{NH}_4)_2\text{SO}_4$ solution, luminescence decayed at a rate that was within the variability of different specimens (fig. 2, B). Unfortunately, the data do not reveal the differences in actual intensity in the $(\text{NH}_4)_2\text{SO}_4$ solution as compared to sea water, although visual observation indicated that it was considerably dimmer in the former than in the latter. In sea water, the decay rate

was greatly increased by the addition of 8 volumes of distilled water (fig. 2, C).

The foregoing observations indicate that the time course of decay is not seriously affected by some physiologically drastic changes in salt content of the medium, nor by dilution with one or more volumes of the same medium (cf. also figs. 5, 6 and 7). With respect to dilution, it will be recalled that

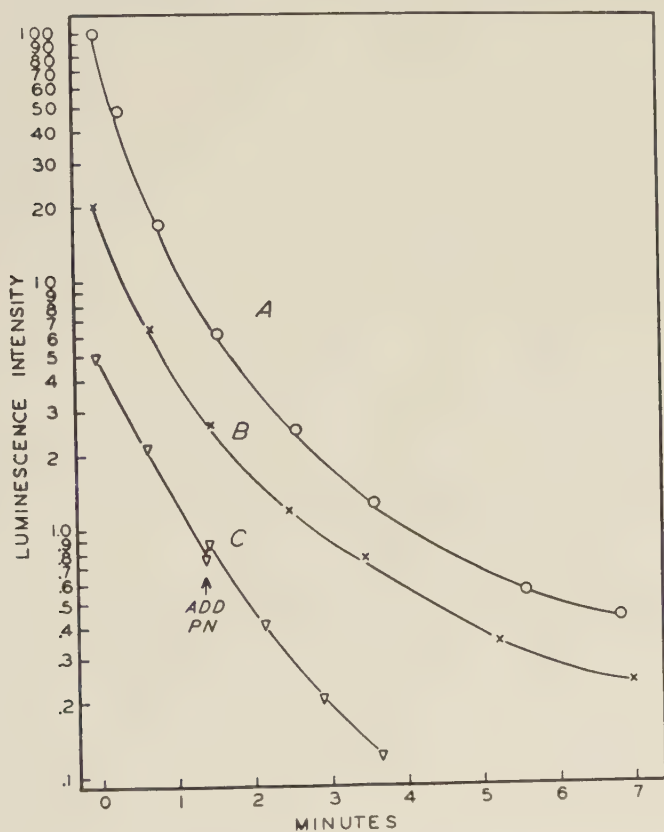


Fig. 1 Rate of decay of luminescence in *Chaetopterus* slime in (A), sea water (one drop of fresh slime added to 1.2 ml sea water); in (B), sea water concentrated 2 X; in (C), phosphate-buffered NaCl solution ("PN"). Arrow on curve (C) indicates the addition of an equal volume of PN. Light intensity is in arbitrary units and each curve is arbitrarily placed with respect to the ordinate in this and succeeding figures, except figure 3.

in these experiments the total volume is kept sufficiently small that the whole solution is exposed to the photo tube. Thus, even though the brightness per unit volume decreased in proportion to dilution, the total light registered at any moment by the photo tube would not be changed by the dilution. The results obtained are similar to those of diluting a suspension of luminous bacteria. In the present instance, if the light intensity were proportional to the concentration of an enzyme in homogenous solution, the rate of decay should drop to one

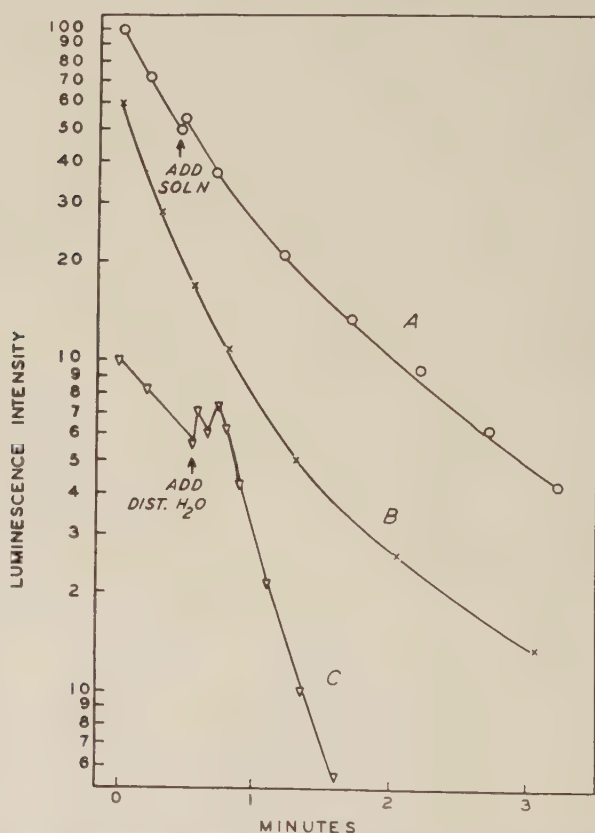


Fig. 2 Rate of decaying luminescence of slime in (A), 0.5 M. NaCl; in (B), nearly saturated ammonium sulfate; in (C), sea water. The arrow on curve (A) indicates addition of an equal volume of NaCl solution, and on curve (C), addition of 8 volumes of distilled water (3.2 ml added to 0.4 ml of slime in sea water).

half on diluting with an equal volume of the medium, or to one fourth if the intensity were proportional to the product of enzyme and substrate concentrations, except with large excess of substrate. Thus, the luminescent material in the slime behaves as if bound to, or within, particles.

Evidence concerning a possible relationship of this behavior to the granules, in presumably photogenic gland cells, revealed in histological studies of *Chaetopterus* (Trojan, '13; Dahlgren, '16; Krekel, '21; Hasama, '41; Bonhomme, '43; Nicol, '52), was not sought in this study. Assuming, in accordance with the results of the dilution experiments, that the reactants are indeed bound together in some manner in particulate form, the changing rate of decaying luminescence, and likewise the variation in kinetics with different specimens, could be logically attributed to an inhomogeneity of the particles. On the other hand, and possibly in addition, the observed kinetics could result from other factors, such as (1), a progressive inactivation, perhaps through thermal instability, of a critical enzyme while acting upon a more stable substrate, or (2), diffusion of a co-factor away from the enzyme, or (3), formation of reaction products that influence the catalytic activity.

With respect to the first of these possibilities, experiments on the collection and storage of the slime in saturated ammonium sulfate and in other media provided some suggestive evidence, as follows. Fresh slime mixed into cold saturated ammonium sulfate solution in an ice bath at -5 to -6°C , glowed with a dim luminescence. When quickly brought to room temperature and diluted with distilled or sea water, the light intensity increased, went through a maximum, then finally disappeared (fig. 3, A-D). The total light produced on dilution was nearly proportional to the amount of slime, while the time course was similar. The time course was changed, however, by first keeping the specimen a short while at room temperature, or for a long time in the deep freeze. At room temperature the rate of rise and fall of luminescence on dilution of the ammonium sulfate solution rapidly decreased,

resulting in a much flatter curve whose total light was difficult to compare with the preceding, but whose time to reach a maximum was lengthened. When stored in the deep freeze at -15°C , the capacity to emit light, when quickly thawed and diluted, persisted for days, but again a flattened curve resulted (fig. 3, C'). These changes are plausibly due to the inactivation of a critical enzyme, through a process that occurs

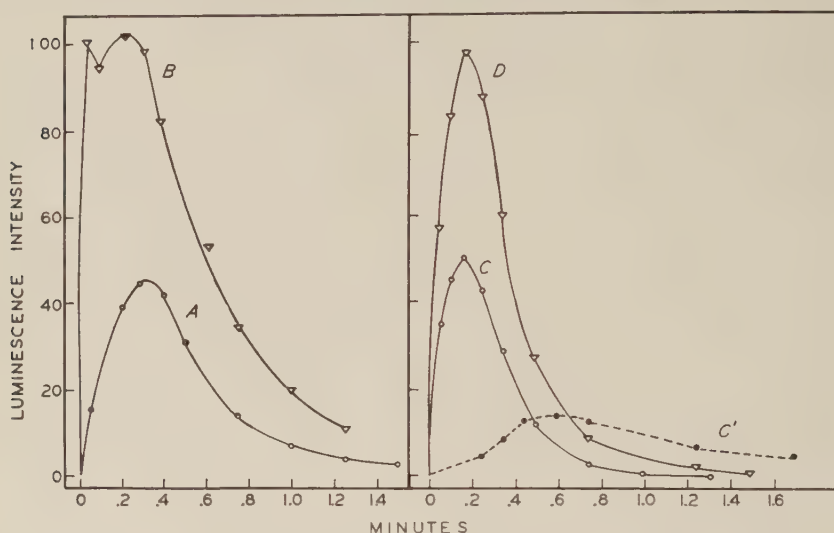


Fig. 3 Course of light intensity following addition of sea water to slime in nearly saturated ammonium sulfate. Curves (A) and (B) are from one specimen and curves (C), (D) and (C') from another specimen. In (A), 2.9 ml sea water were added to 0.1 ml of slime; (B), 8.7 ml sea water to 0.3 ml of slime; (C), 3.4 ml of sea water to 0.2 ml of slime; and (C'), 2.4 ml sea water to 0.1 ml of a portion of the parent specimen of (C) that had been stored three days in a deep freeze at -15°C , then quickly thawed and equilibrated in the water bath.

slowly even at -15°C . It should be mentioned also that slime collected in cold sea water continued to luminesce when held at a temperature as low as -10°C . When transferred to the deep freeze, it retained the capacity for luminescence, with a similar slow rise and fall, on quickly bringing to room temperature. Whole worms placed in sea water and kept in the deep freeze would glow in certain areas for a long time after thawing, though no longer alive.

With respect to the possible role of reaction products, or diffusion away of co-factors to catalytic activity, attempts were made to determine the effects of "spent" slime on the luminescence of freshly secreted slime. Because of the variability in kinetics among different specimens of slime, con-

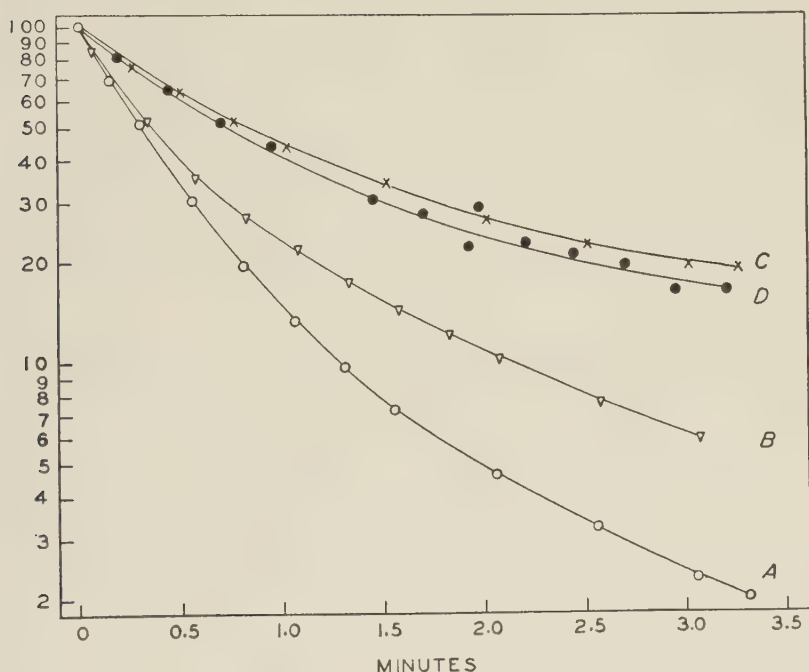


Fig. 4 Kinetics of decaying luminescence in successive specimens of slime from one individual worm, each successive specimen (B, C, D) being added to the accumulated previous specimens. The intensity at the start of recording has been arbitrarily taken as 100 in each case.

clusive results are obviously difficult to obtain. However, figure 4 illustrates the results of one experiment in which 4 successive specimens were obtained from a single individual over a short period of time, and each successive specimen was added to the previous one. Unfortunately, comparisons of initial intensities are not very meaningful; in figure 4 the intensity at the start of recording has arbitrarily been taken

as 100 in each case. Although luminescence in the first specimen decayed faster than the second, and the second faster than the third and fourth, the latter two were about the same. Since the differences in decay rates are not strictly beyond the range of normal variability it cannot be concluded that reaction products in spent slime slow the decay of luminescence of fresh slime. The results indicate the absence, however, of any reaction products in amounts sufficient to increase the rate of decay.

The addition of activated charcoal had no influence on the course of decaying luminescence, except on one occasion when there was reason to believe the sea water had been contaminated with wastes. On this occasion, the addition of small amounts of activated charcoal led to spectacular increases in light intensity.

In view of the foregoing results it appears that an important factor in the decay of luminescence at room temperature is the inactivation of an enzyme. In some instances, the process of inactivation of enzymes or denaturation of proteins has complex kinetics, being rapid at first then proceeding more slowly (cf. Johnson et al., '54), as does the decay of luminescence in the present experiments. If enzyme inactivation is the most important factor in luminescence decay of this material, the difficulty of obtaining a luciferin-luciferase reaction would be largely accounted for.

Influence of co-factors of other systems

Co-factors that are required for, or influence the intensity of, luminescence in other systems were studied with the present system by adding them to the slime both during the course and at the end of decay. Some typical results are illustrated in figure 5. Although in some instances a slight, momentary spurt of increased intensity accompanied the addition, these spurts amounted to scarcely more than those accompanying the addition of sea water alone (cf. also figs. 6 and 7), and are therefore more likely due to mechanical

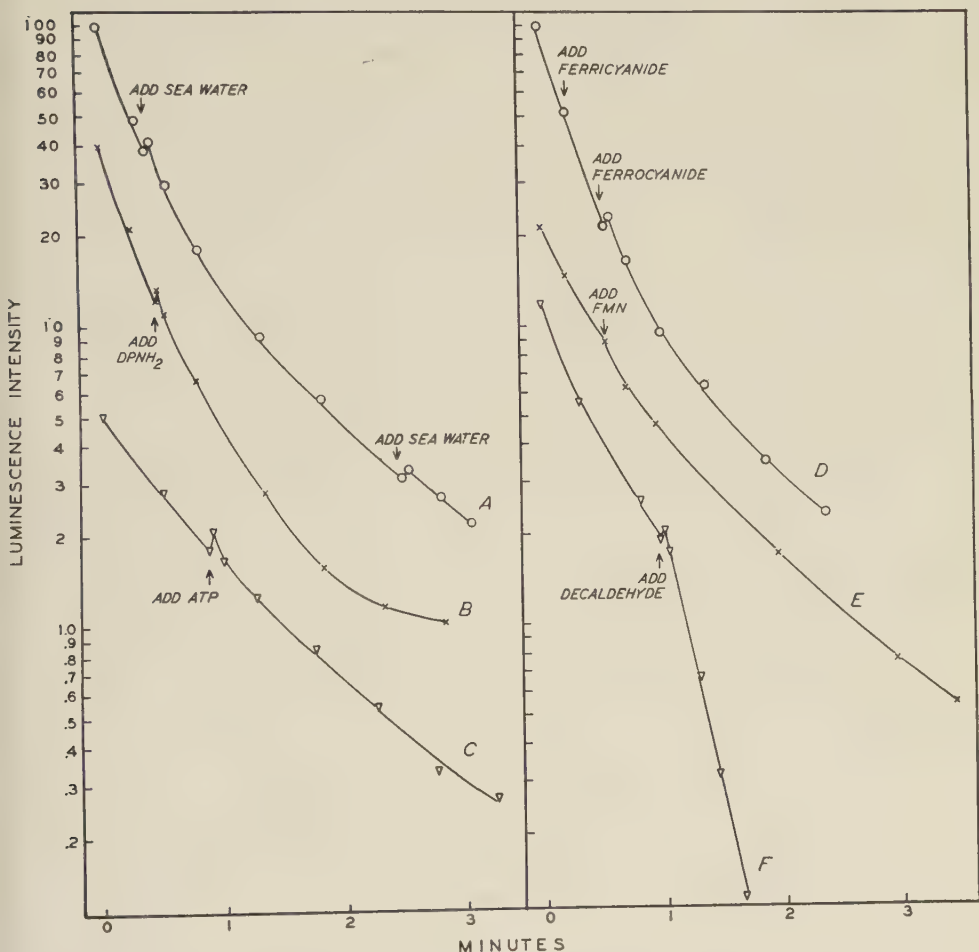


Fig. 5 Influence of adding various solutions, at times indicated by the arrows, on the course of luminescence in sea water. Curve (A), first arrow, 0.5 ml sea water to 0.5 ml slime in sea water; second arrow, additional 2 ml sea water. Curve (B), 0.5 ml sea water containing 1 mg DPNH was added to 0.5 ml of slime specimen. Curve (C), 2 mg ATP in 0.5 ml sea water, neutralized, added to 0.5 ml of slime specimen. Curve (D), 0.5 ml of 0.002 M $K_3Fe(CN)_6$ added to 0.5 ml of slime specimen (first arrow), followed by 1.0 ml of 0.002 M $K_4Fe(CN)_6$ (second arrow). Results were similar when 1.0 ml of 0.01 M $K_4Fe(CN)_6$ was added to 0.5 ml of a different specimen of slime. Curve (E), 0.5 ml sea water containing 0.02 mg FMN was added to 0.5 ml of slime specimen. Curve (F), 0.5 ml sea water containing 0.01 mg decaldehyde (obtained by dilution of 1 ml of a methyl alcohol solution containing 1 mg decaldehyde per ml with 99 ml of sea water) was added to 0.5 ml of the slime specimen.

effects than to any participation in reactions leading to light emission. With the exception of decaldehyde, which functions in light emission in luminous bacterial extracts (Strehler and Cormier, '54) but accelerates the disappearance of luminescence in the present system (fig. 5, F), the substances tested were essentially without influence. They include adenosine triphosphate (ATP), required for luminescence of firefly extracts (McElroy, '47), flavine mononucleotide (FMN) required for bacterial luminescence *in vitro* (Strehler, '53, McElroy et al., '53), reduced diphosphopyridine nucleotide which sustains bacterial luminescence *in vitro* in the presence of FMN and decaldehyde (Strehler '53, '55; Hastings and McElroy, '55), and ferro- and ferricyanide, respectively, which influence the state of Cypridina luciferin that reacts with luciferase in light production (Anderson, '36). Moreover, no combinations of FMN, DPNH and decaldehyde either retarded the decay of luminescence or restored luminescence afterwards. After the light had ceased or nearly ceased, it would not return following the addition of $\text{Na}_2\text{S}_2\text{O}_4$ and vigorous aeration, although such treatment usually results in some further light after decay of luminescence in Cypridina extracts.

Influence of urethane and DFP

Luminescence *in vitro* as well as *in vivo* has long been known to be sensitive to narcotics, and the effects of urethane on bacterial luminescence in particular have been studied at some length (Taylor, '34, '36; van Schouwenburg, '38; Johnson et al., '45, '54; Strehler and Johnson, '54). More recently, di-isopropyl fluorophosphate (DFP) has been found to inhibit the bacterial system reversibly (Johnson and Plough, '59), at higher concentrations than are required for the inhibition of specific cholinesterases in a manner that is irreversible by ordinary methods such as dilution (Bodansky, '46; Webb, '48; Koelle and Gilman, '49, Saunders, '57).

In the present study, addition of appropriate concentrations of either urethane or DFP during the course of the decay

caused a sudden drop in luminescence intensity, which was at least partially reversible on dilution. Typical data are shown in figures 6 and 7, which include also repeated examples showing the absence of any significant effect of adding one to two volumes of sea water alone. The quantitative relation between concentration of inhibitor and amount of inhibition in these experiments is subject to two sources of large error: (1), variations in sensitivity of specimens of slime from different worms or even from the same individual, and (2), the continuous change in luminescence with time, making it difficult to determine the precise extent to which the sudden decreases or increases, following addition or dilution, re-

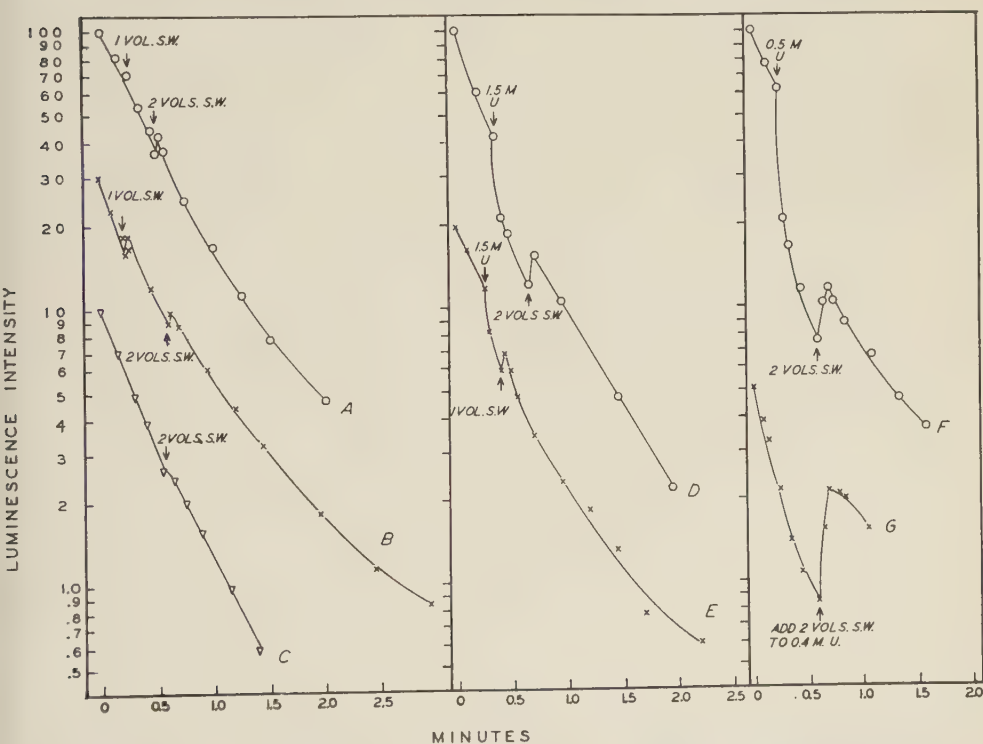


Fig. 6 Influence of adding various concentrations of urethane, and of diluting with sea water, on the course of luminescence decay of slime in sea water, as indicated in the figure. Curve (G) shows the results of adding two volumes of sea water to a slime specimen that had been added initially to a large volume of 0.4 M urethane in sea water.

spectively, measure the effects of the inhibitor. As well as could be estimated by inspection of the curves, 0.075 M to 0.25 M urethane caused sudden inhibitions ranging from 20% to 77%, and 0.002 to 0.0017 M DFP caused sudden inhibitions ranging from 28% to 71%. Averaged values from 25 experiments were plotted on a log-log scale in the manner discussed in detail by Johnson et al. ('54). In spite of some scatter of the points, the relationship for each drug appeared to be linear. The slope of the line drawn by inspection was 1.9 for the urethane inhibition and 0.8 for the DFP inhibition. These values are close to those of 2 and 1, respectively, for the same inhibitors acting on bacterial luminescence at the optimum

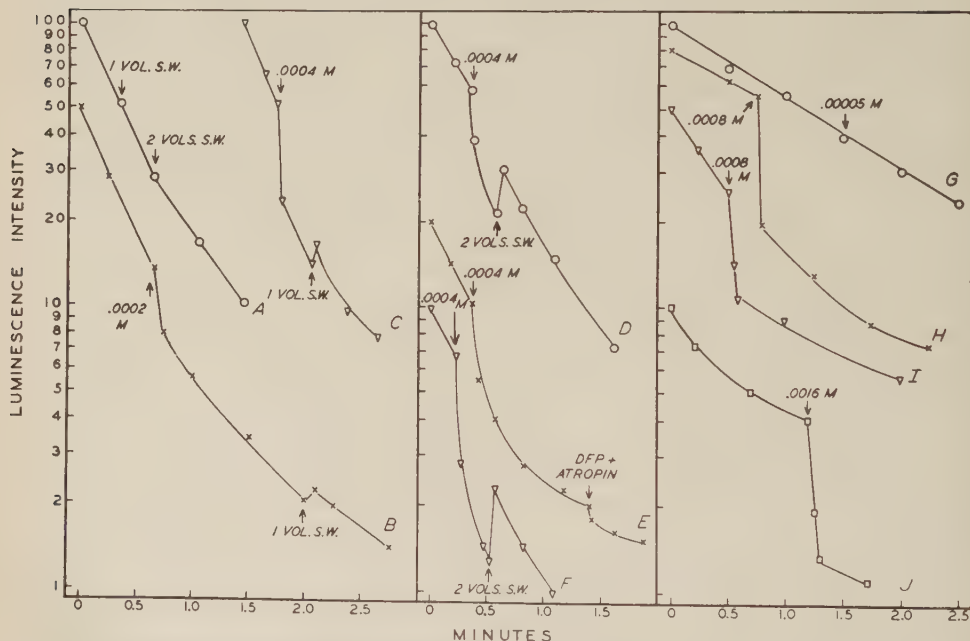


Fig. 7 Influence of adding various concentrations of DFP, and of diluting with sea water, on the course of luminescence decay. Concentrations of DFP indicated on the graph refer to final concentrations after adding 0.5 ml of the inhibitor in sea water to the slime specimen in sea water. In curve (E), a second addition consisted of DFP to give the same final concentration as the first, but in sea water containing also a final concentration of 0.003 M atropin, the well known antagonist of DFP poisoning in mammals.

temperature, and the range of effective concentrations of each drug is also similar.

DISCUSSION

From scarcely more than half a dozen of the myriad types of luminescent organisms, extending among animals from protozoa to fishes and among plants from bacteria to higher fungi, has it been possible thus far to obtain separate, cell-free extracts which emit light on mixing (Harvey, '55). Among the few of which it has been possible, sufficient biochemical information is now available with respect to three, namely, the firefly, bacterial and *Cypridina* systems, to conclude that the different enzymes are specific and the diffusible factors are not the same. Results of the present study indicate that the *Chaetopterus* system is again different, as judged by the failure of diffusible factors important in the firefly and bacterial systems, respectively, to enhance or restore luminescence of the slime. Cold water extracts of the photogenic organs of *Chaetopterus* have been found to give no visible light with *Cypridina luciferin* (Harvey, '52) or on adding ATP (Harvey, '49; Haneda and Harvey, '52).

Using the secreted slime as starting material, rather than the entire photogenic organs, a number of unsuccessful attempts were made in this study to separate components active in light emission. Although a detailed account would not seem justified, the classical method of testing for a luciferin-luciferase reaction was one of the methods tried, i.e., brightly luminous slime was quickly heated to the point that no light returned on cooling, and was then added to spent slime, but no light appeared. Some encouraging but irregular results were obtained by collecting the slime in cold acetone, filtering off the precipitate that formed and drying the acetone soluble extractives to a faintly yellowish, greasy and salty residue. When a 3% solution of this residue was added in small amounts during the course of luminescence decay of slime in sea water, large increases in light occurred in some instances

but only very slight increases in others. Wetting the residue with sea water, or adding 3% solutions of the residue to solutions of the precipitate that had been filtered out of the acetone and then dried, did not result in detectable light emission, and the dried precipitate itself gave no light when wet with sea water.

The kinetic evidence that the reactants in luminous slime are organized in a particulate manner, rather than occurring in homogenous solution, suggests that this may be an important factor in the difficulty of separating active components. The evidence for instability of an essential catalyst suggests another factor, perhaps even more important. The method, however, of using the kinetics of decay as a means of assay for the influence of various factors, and of investigating the properties of such systems as this, apart from the added complexities of physiological control, appears to offer further possibilities (cf. Sie, Chang and Johnson, '58³). By appropriate refinements in technique, the slime could probably also be used satisfactorily for investigating the action of factors influencing the total, as well as instantaneous, intensity of the emitted light.

SUMMARY

The kinetics of decreasing light intensity in the luminous slime secreted by *Chaetopterus variopedatus* has been studied with reference to the order of decay and the influence of salt content of the medium, diffusible factors of other luminescent systems, and the inhibitory effects of urethane and DFP.

The initial rate of decay was nearly exponential, but decreased progressively, sometimes ending in a dim, long-lasting glow of luminescence.

The time course of decaying luminescence of slime in sea water was not significantly altered by aeration with air or oxygen, by addition of one or two volumes of sea water, by addition of small concentrations of FMN and DPNH, separately or in combination, or by addition of ATP. The decay

³ See footnote 3, page 259.

rate was increased by adding small concentrations of decaldehyde or 8 volumes of distilled water.

The kinetics of decaying luminescence of slime added initially to twice concentrated sea water, to 0.5 M NaCl, to 0.25 M NaCl in 0.125 M phosphate buffer of pH 7.3, or to saturated ammonium sulphate was essentially the same as that of slime added initially to sea water. The relatively dim luminescence of slime added initially to saturated ammonium sulphate brightened on dilution with sea water, then decayed, giving a total light approximately proportional to the amount of slime, in different portions of the same specimen kept cold. Light-emitting capacity of slime mixed into saturated ammonium sulfate solution was quickly lost at room temperature, but could be preserved for days at -15°C , accompanied by a slowing of the rate of increasing intensity when quickly thawed and diluted. Quickly frozen fresh slime retained a capacity for light emission on thawing after storage at -15°C , as did whole worms.

During the course of decaying luminescence of slime in sea water, sudden decreases in intensity occurred on the addition of urethane in final concentrations of 0.075 to 0.25 M, or of DFP of 0.0002 to 0.0017 M. Sudden increases in intensity then occurred on dilution with an equal or larger volume of sea water.

Attempts by various methods to obtain, from luminescent slime, components active in light emission led to negative or equivocal results.

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THE ACTION OF DFP AND RELATED DRUGS ON BACTERIAL LUMINESCENCE ¹

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FOURTEEN FIGURES

The properties of fluorophosphonate (fluorophosphate, phosphofluoridate) compounds and their cogeners as nerve poisons, insecticides, enzyme inhibitors and, in some measure, therapeutic drugs, have led to extensive research and a fairly voluminous literature that has grown up chiefly since the close of World War II (cf. Saunders, '57; Sollmann, '57; Goodman and Gilman, '55). The extraordinary toxicity of these compounds, of which di-isopropylfluorophosphonate (DFP) has been one of the most widely studied, is usually attributed to their anticholinesterase activity which, according to results with *in vitro* preparations and other evidence, involves a combination of the drug with the enzyme in a manner that is not reversible by ordinary methods (Macworth, '42; Bodansky, '46; Jandorf et al., '55), in this respect differing from the action of numerous other cholinesterase inhibitors, such as eserine (Koelle and Gilman, '49).

In addition to the "true cholinesterase" of nerve and red blood cells and the "pseudocholinesterase" of plasma or serum (Mendel and Rudney, '43), which undergo inhibition by fluorophosphonates in concentrations of the order of 10^{-5} to 10^{-11} molar (cf. table and figure, pp. 489-493, in Johnson,

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Eyring and Polissar, '54), certain other enzymes, including other esterases, proteinases and dehydrogenases, are subject to inhibitions, with varying degrees of sensitivity (cf. Jansen et al., '48; Webb, '48; Michaelis, Arango and Gerard, '49; Balls and Jansen, '52). Moreover, respiration of brain and nerve is inhibited in drug concentrations that also affect electrical activity (Brooks, Ransmeier and Gerard, '49). Although the evidence indicates that the acute toxicity displayed by these drugs is due to the inhibition of true cholinesterases, the total physiological effects may include action on oxidative and other systems also.

The present study was undertaken in an effort to contribute to an understanding of the general mechanism of action of DFP and other drugs of related interest, with particular reference to oxidative reactions in cells. For this purpose, the luminescent system of bacteria provides unusually favorable material from several points of view: (1), its unique advantage of having a natural indicator, viz., brightness of emitted light, of the over-all reaction velocity, which can thus be instantaneously and accurately measured in relative units; (2), a system that can be easily obtained and has fundamentally the same steady state properties, in cell-free extracts as well as in suspensions of intact cells (Strehler, '53, '55; Strehler and Cormier, '53; Strehler and Johnson, '54; Hastings and McElroy, '55); an *in vitro* system wherein by adjusting the concentrations of the diffusible factors involved, different steps in the sequence of reactions leading to light emission can be made rate-determining; and (4), a system that has, among different species of bacteria, each of whose luminescent system uses the same diffusible factors, different "normal" temperature-activity curves. This last mentioned feature is particularly useful with reference to the temperature relations of a drug's action, which cannot be fully understood apart from the temperature-activity curve of the system affected (Johnson, Eyring and Polissar, '54).

MATERIALS AND METHODS

Three species of marine luminous bacteria were employed in this study: *Photobacterium phosphoreum*, *Achromobacter fischeri*, and *Achromobacter harveyi*, having optimum temperatures for luminescence of cells, suspended in buffered salt solution from fully grown cultures, of approximately 22°, 28° and 32°C, respectively (Johnson, Eyring and Williams, '42). The optimum temperatures for growth and development of brightly luminous cultures are somewhat lower, being 15° to 17°C for the psychrophile, *P. phosphoreum*, and 20 to 25°C for the other two species. Cultures of *P. phosphoreum* and *A. fischeri* were made on ordinary nutrient agar with the addition of 3% NaCl, 0.5% powdered CaCO₃, and 0.5% glycerol. The medium for *A. harveyi*, containing fish extracts to enhance luminescence (Johnson and Shunk, '36), consisted of equal parts of nutrient broth in 3% NaCl and of filtered extractives of 1 lb. of flounder boiled in distilled water plus 0.5% NaCl, with the addition of 1.8% Bacto-agar to the final solution.

After approximately one day's incubation, the brightly luminous cells were suspended in a large volume of "PN" solution, consisting of equal parts of 3% NaCl and 0.25 M Na₂HPO₄-KH₂PO₄ buffer, pH 7.0. Aliquot portions were added to PN solution, or PN containing different concentrations of DFP, in standard, flat-bottomed Hellige colorimeter tubes, capacity about 10 ml. The same tubes were used for reaction mixtures of the extracted enzyme, prepared as described below.

From each species, crude enzyme extracts were prepared by acid precipitation from the supernatants of distilled water cytolysates of washed cells, according to the method of McElroy et al. ('53, '54) and were stored in a deep freeze. For sustained luminescence, the reaction mixture ("saturated system") usually consisted of a portion of the stock enzyme preparation added in a ratio of one part of enzyme solution to 50 to 100 parts of 0.1 M phosphate buffer, at pH 7.0, or buffer plus DFP, containing final concentrations of 1 mg DPNH,

2 μ g FMN, and 20 μ g of decaldehyde per milliliter, in a final volume of 4 ml.

Luminescence was measured by a RCA 1P21 photomultiplier, whose amplified signal was fed into an Esterline-Angus automatic recorder. The specimens of luminous bacteria, or

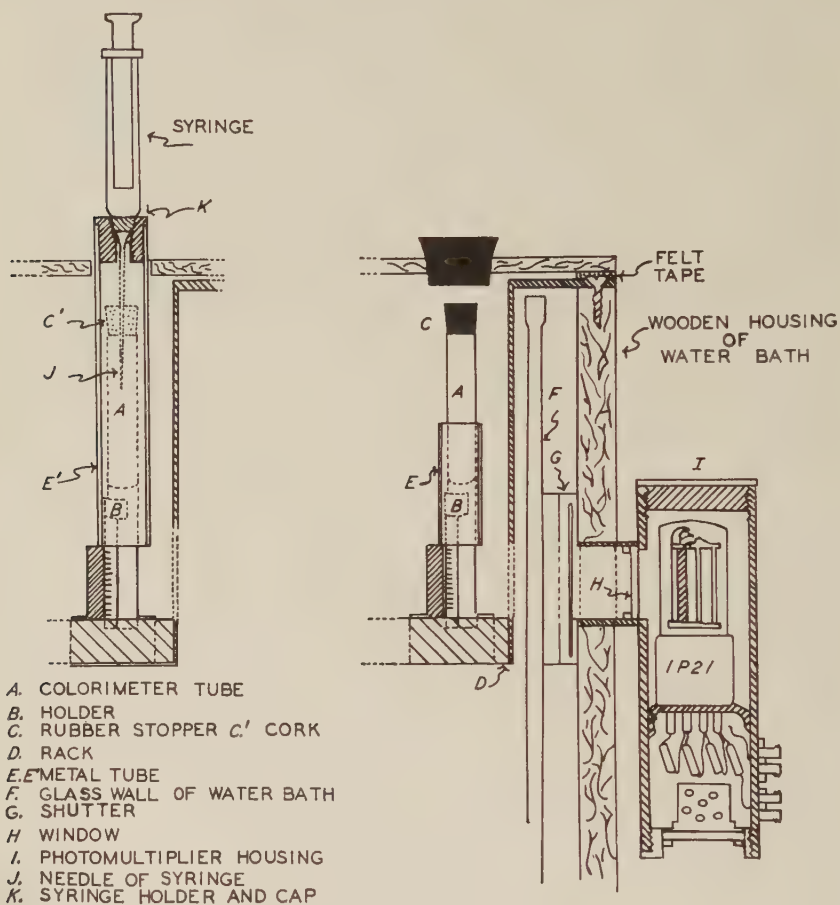


Fig. 1 Diagram of arrangement of specimen tubes in a constant temperature water bath, enclosed in a black wooden box with hinged lid, for recording the intensity of luminescence passing through a window and shutter to a photomultiplier. The cut-away diagram at the left shows the arrangement for adding a solution from a syringe to the specimen in a standard, colorimeter type glass tube, without exposing the photo tube to extraneous light.

luminescent extracts, in colorimeter tubes were held in a water bath at constant temperature, shielded from extraneous light by a wooden housing (fig. 1), and any given tube was exposed to the photo cell by placing in a holder (fig. 1, B) and opening the shutter (fig. 1, G) after closing the lid of the bath. A metal sleeve (fig. 1, E or E') extended well below the meniscus of the luminous specimen. The arrangement indicated at the left in figure 1 was used when it was desired to introduce a solution from a syringe without interrupting the course of recording of the light. A cork with a small hole drilled through the center (fig. 1, C') was then used as the tube closure, rather than the usually employed rubber stopper (fig. 1, C). With either arrangement, a constant area of the specimen tube was exposed to the phototube, and the volume of the specimen was always more than sufficient to fill this area. Thus, on diluting a suspension of bacteria (provided the suspension was not very thick) with an equal volume of PN, the light intensity dropped to one half, since the brightness per unit volume decreased in proportion to concentration of cells per unit area exposed to the photo tube. On the other hand, if the suspension contained a luminescence inhibitor whose action was reversible by dilution, the light would fail to decrease with dilution by the amount that the inhibition was reversed. With the extracted enzyme system, rather than whole cells, the results of dilution were complicated by the fact that the intensity of luminescence is not directly proportional to the concentration of enzyme, especially at very low enzyme concentrations, even with the diffusible factors in excess (Strehler and Cormier, '53).

Bacterial suspensions were usually made thin enough that the dissolved oxygen, essential for light emission, was not readily exhausted. Over considerable periods of time, however, it was necessary to aerate the suspensions, by removing from the bath and inverting the tube several times. The same procedure was used with the extracted enzyme solutions. Vigorous shaking of the latter was found to impair luminescence, possibly by surface denaturation of the enzyme.

RESULTS OF EXPERIMENTS

Reversible DFP inhibition of luminescence in cells

Preliminary experiments showed that the intensity of luminescence quickly dropped on the addition of luminous bacterial cells to DFP solutions in concentrations of the order of 10^{-4} to 10^{-2} molar, and this initial drop underwent rapid reversal on dilution. Figure 2 shows the results of an experiment with whole cells of *A. fischeri* (cf. also figs. 3, 4, 7-9).

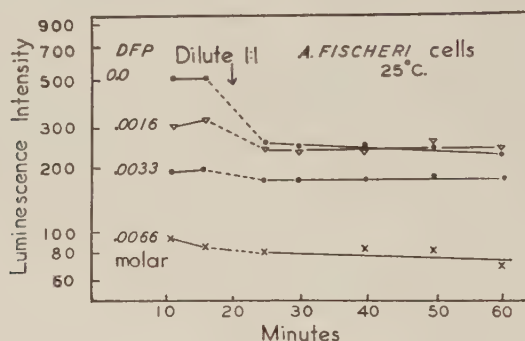


Fig. 2 Reversibility of the DFP inhibition of luminescence in *A. fischeri* cells by dilution with an equal volume of salt solution. The intensity per unit volume in the control drops to one half on diluting the cell density to one half, whereas with DFP concentrations of 0.0033 or 0.0066 molar, that cause considerable inhibitions, the recorded intensity remains the same, the average light intensity per cell doubling as the cell density is halved by the dilution.

Dilution of a drug-free control with an equal volume of salt solution resulted in a 50% reduction in luminescence intensity. Less reduction occurred in a corresponding suspension containing a slightly inhibitory concentration of DFP (0.0016 M), and essentially no change occurred on dilution of the suspensions with the more strongly inhibitory concentrations (0.0033 and 0.0066 M, respectively). It follows that with the latter concentrations, the average light intensity per cell²

² The unlikely alternative, that DFP acts in an all-or-none manner on the luminescence of individual bacterial cells, is not easily excluded, but the evidence for reversibility would be the same since, in that situation, previously dark cells would have to luminesce to account for the effects of dilution.

increased in proportion to the decrease, by dilution, in number of cells per unit volume, and the inhibition, therefore, was reversed in proportion to dilution.

TABLE 1

Influence of density of cell population of A. fischeri on the amount of inhibition by 0.00125 M DFP at 8° to 9°C

A suspension of cells was diluted with salt solution, added to a buffered salt solution with or without DFP at room temperature, and placed in the water bath at 8° to 9°C. Readings of luminescence intensity were made at approximately three, 4, and 5 minutes after adding the cells.

RELATIVE DENSITY OF CELLS	TIME AFTER MIXING	LUMINESCENCE INTENSITY		PER CENT INHIBITION BY DFP
		Control	DFP	
	<i>minutes</i>			
1	3	27.5	8.5	69
	4	25.0	7.5	70
	5	23.0	7.5	67
1/4	3-4	9.0	2.0	78
	5	8.5	2.0	76
1/8	2.4	5.5	1.3	76
	3.4	4.9	1.1	78
	4.4	4.9	1.0	80

The initial, reversible inhibition was found to be relatively independent of concentration of cells. Table 1 gives the data from an experiment done at a low temperature to avoid certain complications that occur at the higher temperatures, as discussed presently. These data show that in spite of an 8-fold difference in density of cells, the per cent inhibition varied only between 67 and 80%.

Influence of DFP concentration and temperature

At temperatures low with respect to the normal optimum for luminescence of a given species, a lower concentration of DFP was required for a given initial inhibition. The amount of inhibition at various concentrations, however, had a tendency to change with time, usually becoming less as time went

on, except with the highest concentrations which resulted in an inhibition that became greater with time. These results are illustrated in figures 3, 4 and 5, the first two of which show the effects also of dilution and of changing the temperature. The effects of dilution correspond to the results shown in figure 2, whether the dilution is made before or after the change in temperature.

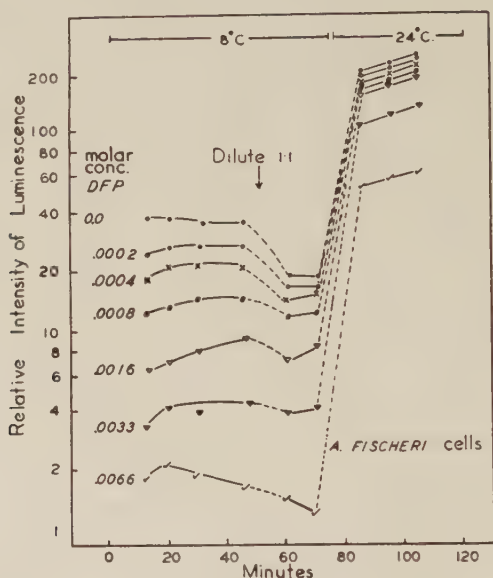


Fig. 3 Influence of temperature and of dilution on the inhibition of luminescence in cell suspensions of *A. fischeri* by various concentrations of DFP.

The effects of raising the temperature depend upon the concentration of DFP involved, which for convenience of discussion may be referred to as low, roughly from 0.0002 to 0.004 M, and high, 0.01 M or more, respectively. Within the former range, a marked decrease in per cent inhibition occurred on changing from a cold to higher temperature, provided the normal optimum temperature was not exceeded. With the latter, i.e., the high concentrations, the initial effect of similarly raising the temperature was again to reduce the amount of inhibition, but in this case was followed by a pro-

gressive increase in inhibition on keeping at the higher temperature. Converse effects were obtained on changing the temperature in the opposite way from the relatively high to low; i.e., the inhibitory effects of low concentrations were increased, and the rate of progressively increasing inhibition caused by high concentrations was lessened. Evidently, the

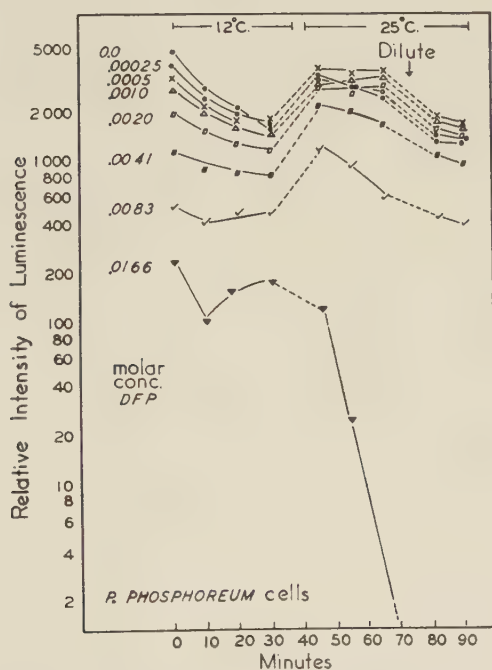


Fig. 4 Influence of temperature and of dilution on the inhibition of luminescence in *P. phosphoreum* cells by various concentrations of DFP.

inhibition caused by a high concentration involves two actions, one of which represents a mobile equilibrium, which changes reversibly with temperature or with drug concentration, and the other a rate process whose rate is influenced by the same factors, but is conspicuous only at the high concentrations and is accentuated with rise in temperature. This latter process has a high temperature coefficient, as shown by the precipitous drop in intensity of luminescence in the presence of 0.0166 M

DFP at a temperature of some 10° or 11°C above the normal optimum, (fig. 6; compare with fig. 4). The loss in activity with time of incubation is not restored on cooling to the optimum temperature, and thus represents a thermal destruction of an essential component of the system, catalyzed by DFP. The high temperature coefficient indicates an enzyme as the

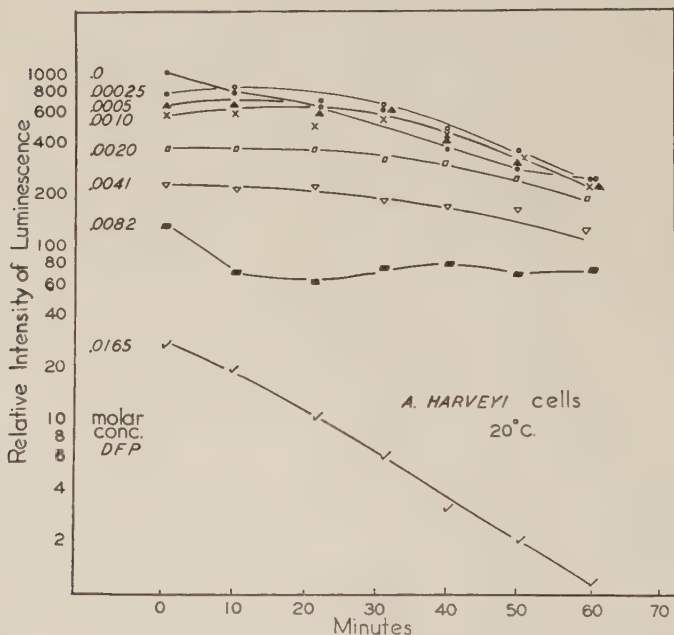


Fig. 5 Inhibition of luminescence in *A. harveyi* cells by various concentrations of DFP at constant temperature.

component affected. Further evidence is provided by the data illustrated in the inset of figure 6, pertaining to the extracted enzyme maintained in aliquot portions at 33°C , with and without DFP, for successively longer periods of time, then tested for activity at 25.5°C , showing a faster loss of catalytic activity in the presence of DFP.

Analysis of the relation between DFP concentration and amount of initial inhibition, according to the formulation applicable to reversible inhibitions at a given temperature and

pressure (Johnson, Eyring and Polissar, '54), is shown in figure 7. The relation is linear for *P. phosphoreum* at 12° and for *A. fischeri* at 8° and 25°C, with slopes just slightly more than unity, indicating that the drug-enzyme combination occurs in a molecular ratio of one to one.

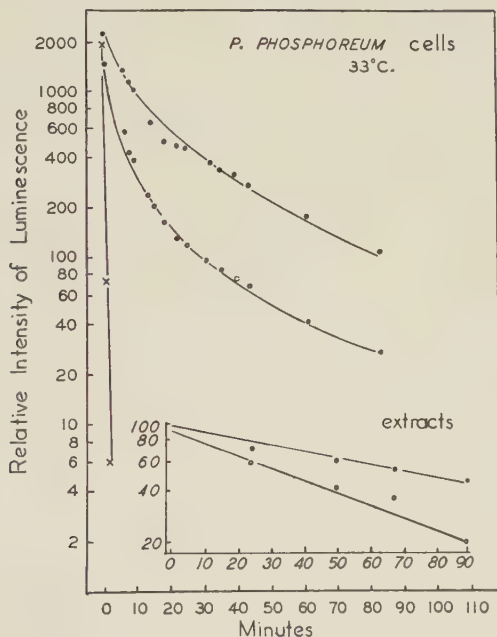


Fig. 6 Acceleration of the thermal destruction of the luminescent system of *P. phosphoreum* by 0.0041 M (open circles) and 0.0166 M (crosses) DFP in cells and (inset) by 0.0057 M DFP (open circles) in crude enzyme extracts. The inset shows the percent activity remaining after incubating aliquot portions of the enzyme solution, with and without DFP, for the successive periods of time indicated on the abscissa, then cooling to 25.5°C, and assaying for light-emitting activity in 7½ volumes of reaction mixture.

The curved line pertaining to *A. harveyi* (fig. 6) has several possible interpretations. Linear, as well as similarly curved, lines have been encountered in analyzing, in the same manner, the DFP inhibition of cholinesterase of horse serum, and of human and rabbit serum, respectively (Johnson, Eyring and Polissar, '54, p. 493). Although the problem was not pursued further in the present study, two plausible, alter-

native but not mutually exclusive interpretations of the curved line are (1), combination of DFP with an inert constituent in the bacterial suspension, thereby altering the effective concentration so that this concentration is no longer proportional

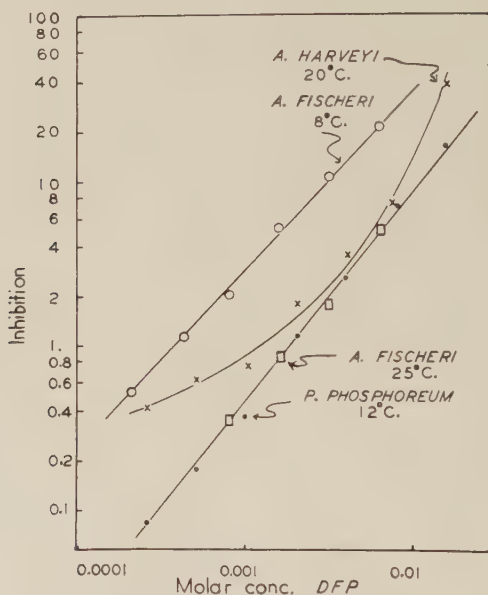


Fig. 7 Analysis of the relation between concentration of DFP and amount of initial, reversible inhibition of luminescence in cells of the three species of luminous bacteria studied. On the bottom, right hand line, circles are for *P. phosphoreum* at 12°C, and rectangles for *A. fischeri* at 25°C. The amount of inhibition is expressed on the logarithmic scale of the ordinate as the intensity of the control (I_c) divided by the intensity at a given concentration of DFP (I_{DFP}) minus one.

to the amount added to the suspension (Johnson, Eyring and Kearns, '43), or (2), multiple inhibitory reactions of the drug on a single, or more than a single essential enzyme, as in the action of urethane (Johnson, Eyring and Polissar, '54).

Action of Sarin on cellular luminescence

The considerably more toxic ester, Sarin (isopropyl methyl-fluorophosphonate), inhibits bacterial luminescence at the

same range of molar concentrations as DFP, though in terms of milligrams per ml the concentration is less because of the lower molecular weight. The mechanism of action is also similar to that of DFP, in regard to reversibility by dilution and

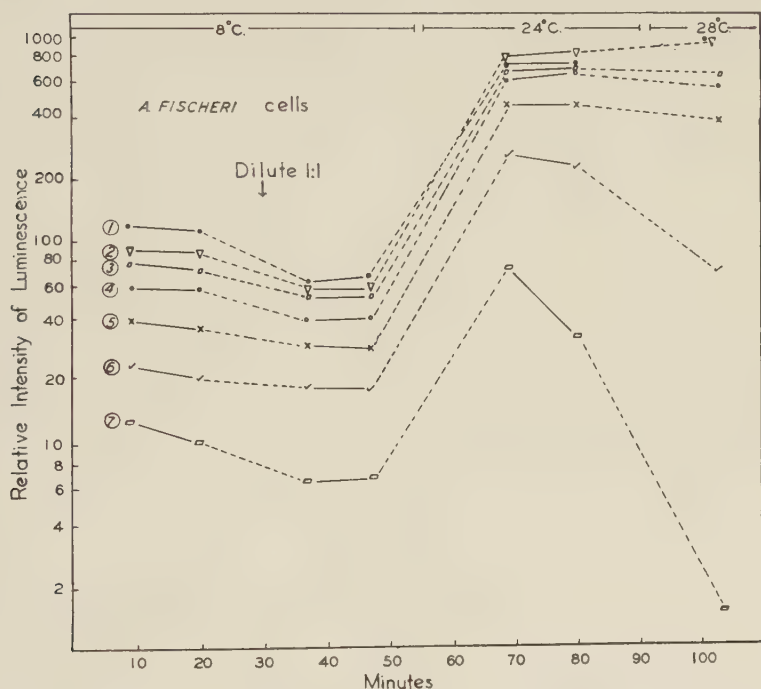


Fig. 8 Influence of dilution and of temperature on the inhibition of luminescence in cells of *A. fischeri* by various molar concentrations of Sarin, in curves numbered 1 to 7, as follows: 0, 0.00045, 0.0009, 0.0018, 0.0035, 0.007, and 0.014, respectively.

the influence of temperature, as illustrated in figures 8 and 9, except that, with high concentrations, the increase in rate of irreversible inhibition at higher temperatures is more pronounced. This added inhibitory action at the higher temperatures is responsible in part for the increase in slope of the line, at 24° as compared to 8°C, relating the amount of inhibition to concentration of the drug (fig. 9).

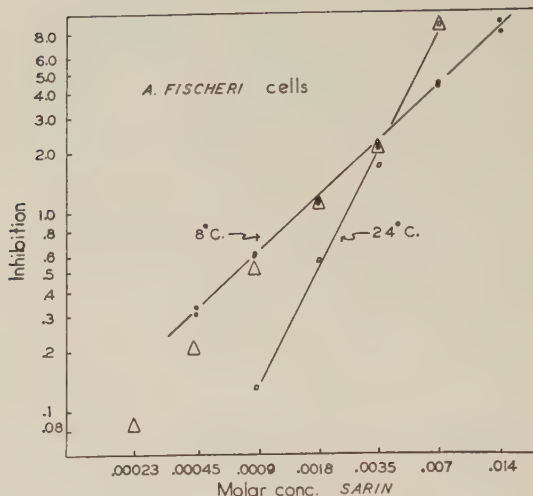


Fig. 9 Analysis, in the manner of figure 8, of the relation between concentration of Sarin and amount of inhibition, according to the data from figure 8. Circular points, for the line at 8 degrees, are from repeated readings of intensity shortly after adding the Sarin, and triangles represent the amount of inhibition after a 1:1 dilution with salt solution. Rectangles are from the data at 24°C.

Influence of DFP and Sarin on luminescence of cell-free extracts

Luminescence in the extracted system was found to be much less sensitive to inhibition by DFP than in whole cells, about 10 times the concentration being required for corresponding inhibitions, possibly because of the presence of considerable amounts of inert substances that bind DFP in the crude extracts. The same is qualitatively true of the action of urethane and of sulfanilamide on this system (Strehler and Johnson, '54). Also, in the action of DFP on crude and partially purified acetylcholinesterase only one tenth the concentration is required for a 50% inhibition of the purified as of the crude enzyme preparation (Jansen et al., '48). The luminescence of extracts at optimum temperature gradually decays as a first order reaction under the conditions of the present experiments, making it somewhat less convenient than whole cells in a study of this kind. With a high concentration of

DFP, such as 0.04 M, required for a considerable inhibition at the start, the decay rate is greatly increased (fig. 10) probably because the drug catalyzes the destruction of an essential enzyme (cf. fig. 6). With less strongly inhibitory concentrations, 0.005 to 0.01 M, the decay rate changes with time, for reasons that are not clear. Approximate values for the amount

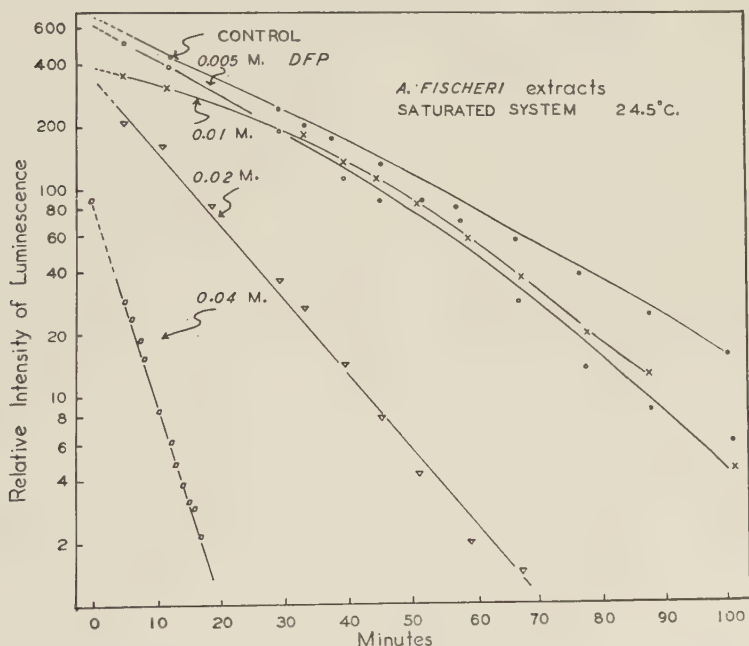


Fig. 10 Influence of high concentrations of DFP on the luminescence of *A. fischeri* extracts.

of initial, presumably reversible inhibition can be obtained by extrapolating (dashed lines in figure 10) the decay lines back to zero time, which in these experiments was the time of adding the enzyme to the remainder of the reaction mixture containing various concentrations of DFP, kept in an ice bath until just before using. When such data were plotted in the manner of figures 7 and 9, an approximately linear relation resulted, with a slope of 2.

At low temperatures, no increase in potency of DFP was observed with the extracted system, in marked contrast to the results obtained with cells. In fact, at 16°C, slight increases in the intensity of luminescence occurred in specimens containing 0.0016 to 0.0066 M DFP. When the same specimens

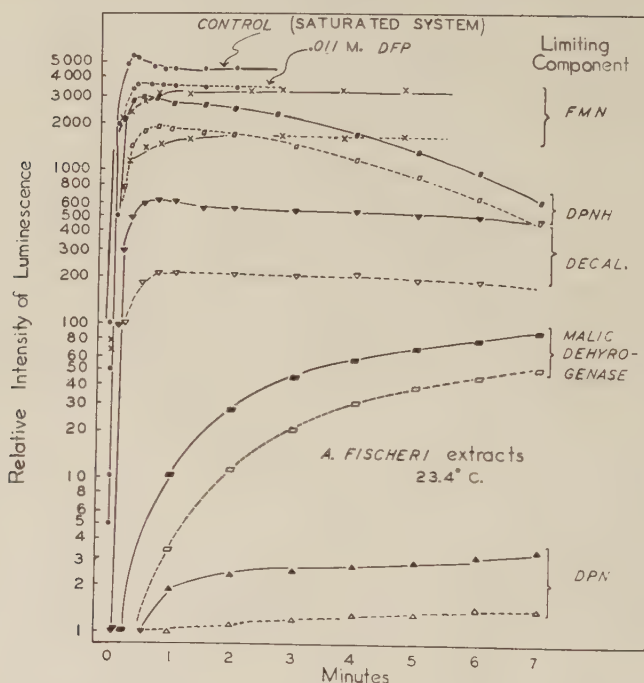


Fig. 11 Influence of DFP on the luminescence of *A. fischeri* extracts with different components limiting the intensity of light emission, as described in the text.

were brought to 25.5°C, no inhibition was evident at first, but after 45 minutes the luminescence of the tube containing 0.0066 M DFP was about one half as bright as the control. It appears, therefore, that a component or factor sensitive to DFP in intact cells is either practically lacking, or for unknown reasons without significant influence, on the luminescence of extracts.

With any one of the diffusible components that function in the extracted system reduced in concentration to the point that it limits the light-emitting capacity of the system, the inhibitory effect of DFP at the optimum temperature remained about the same. Figure 11 shows the results of an experiment wherein the concentrations of DPNH and decaldehyde, respectively, were only one one-hundredth, and the concentration of FMN only one two-hundredth of the amounts usually employed to give a maximum, sustained luminescence, the reduced concentrations of only one of these components being used at a time. The course of rising intensity with DPNH added in either excess or low concentrations was always too rapid to measure accurately by the method available. The bottom 4 curves show the much slower course of rising intensity without DPNH added as a hydrogen donator, depending instead on the activity of the malic dehydrogenase present in the crude extracts, with and without DPN in a rate-limiting concentration. The intensity of sustained luminescence was reduced by 0.011 M DFP in each case, to extents ranging from about 30% with the lowered concentrations of DPNH, to about 60% with the lowered concentrations of decaldehyde or DPN. No clear distinction can be made, on the basis of these results, between the sensitivity to DFP inhibition of the several reaction steps.

The action of Sarin was found to be generally similar to that of DFP, except that the concentrations effective for inhibiting the luminescence of extracts at the optimum temperature were essentially the same as for the luminescence of whole cells. As with DFP, the amount of inhibition caused by a given concentration of Sarin was much less at low temperatures (fig. 12). The destructive action evidenced by the increase in amount of inhibition with time, however, was more strongly dependent on temperature and concentration. For example, the high concentration of 0.014 M Sarin caused a slight, constant reduction in luminescence intensity at 8.4°C (fig. 12), but on warming to 16°C it caused a rapid destruction. At half this concentration, the same rise in temperature

resulted in virtually no increase in amount of inhibition, but on warming further to 26°C, rapid destruction ensued.

The temperature relations revealed in figure 12 suggest the possibility that the resistance of bullfrogs to Sarin at

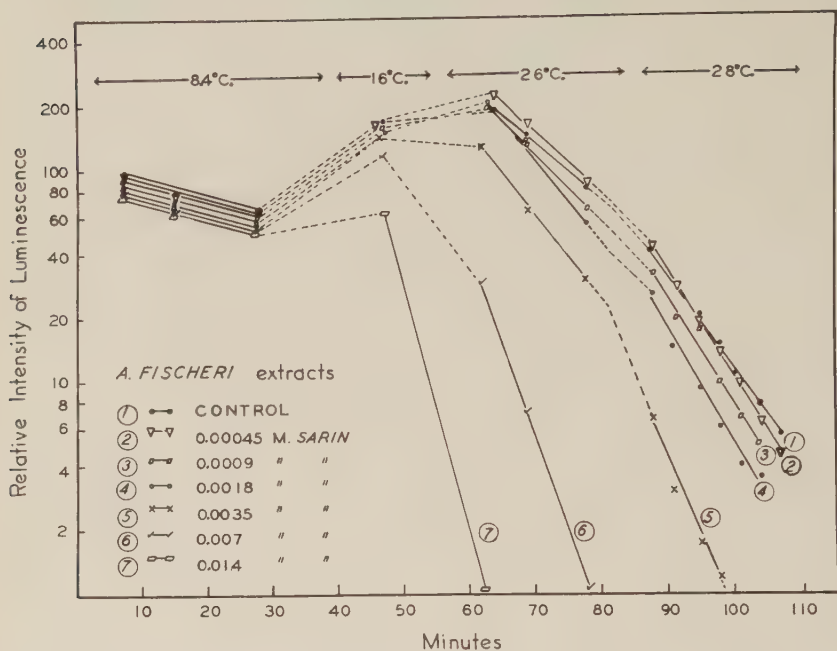


Fig. 12 Influence of Sarin on the luminescence of *A. fischeri* extracts. Sarin concentrations are the same as those in figure 8.

22°C (Wilber, '54) is due in part to similar relations in the toxic action of Sarin on cold-blooded animals. Moreover, these relations suggest that a decreased toxicity might be reasonably expected in states of hypothermia in mammals, and conversely, an increase in toxicity at elevated body temperatures.

Action of eserine, fluoride, and isopropyl alcohol

In respect to the range of inhibitory concentrations and reversibility on dilution, the action of eserine on the luminescence of whole cells was essentially the same as that of DFP

and Sarin, but there were two differences in the effects of temperature (fig. 13). First, at no concentration was the amount of inhibition greater at a low temperature than at the optimum. Instead, each concentration caused a slightly greater inhibition at the optimum than at a lower temperature. Second, at high concentrations, there was no evidence of a significant increase in amount of inhibition with time of exposure to the drug.

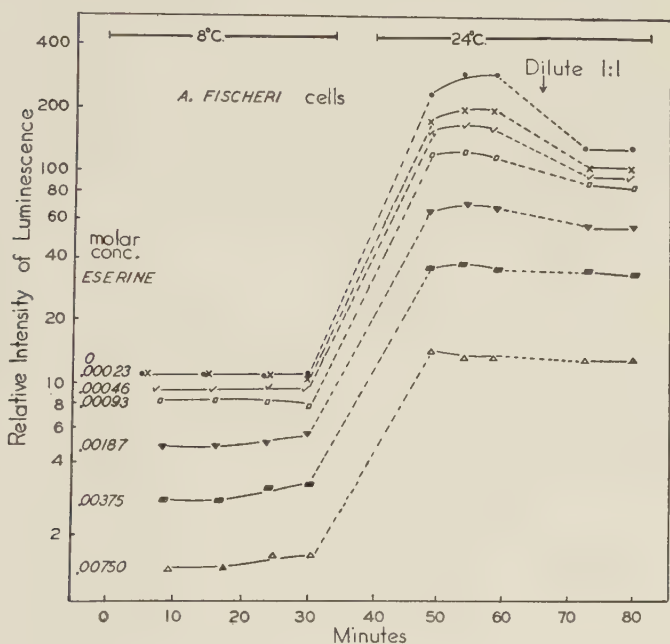


Fig. 13 Influence of temperature and dilution on the inhibition of luminescence of *A. fischeri* cells by various concentrations of eserine.

The concentrations of fluoride and isopropyl alcohol, respectively, that had to be added in order to produce any considerable inhibition of luminescence in either cells or extracts, were high, of the order of tenths molar for fluoride and, depending on the temperature, somewhat higher for the alcohol. It follows that the DFP inhibition is not caused by free fluoride or isopropyl alcohol liberated from the drug.

The fluoride inhibition was not studied in detail. Experiments on the temperature-dependence of the isopropyl alcohol inhibition revealed that pronounced increases in luminescence intensity at low temperatures were caused by certain concentrations which resulted in pronounced decreases in intensity at the optimum temperature. A similar phenomenon

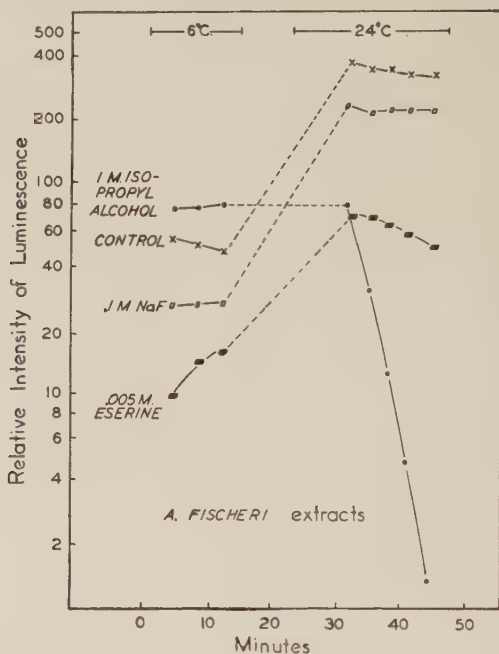


Fig. 14 Inhibition of luminescence of *A. fischeri* extracts by selected concentrations of isopropyl alcohol, fluoride, and eserine, respectively, at two temperatures.

has been observed in the effects of ethyl alcohol on the luminescence of both cells (Johnson et al., '45) and extracts (Strehler and Johnson, '54). Luminescence in whole cells is more sensitive to either ethyl or isopropyl alcohol than in crude extracts.

The influence of temperature on the effects of selected concentrations of fluoride and isopropyl alcohol, respectively, on luminescence of *A. fischeri* extracts is illustrated in figure 14,

which includes also data with respect to eserine. A very high temperature coefficient for the inhibitory action of isopropyl alcohol is evidenced by the change from a stimulative effect at 6°C to a rapidly destructive effect at 24°C. In line with the kinetic analyses of the action of alcohol and urethane (Johnson et al., '54), the rapid drop in luminescence intensity at 24°C most likely represents the catalysis of enzyme denaturation by isopropyl alcohol.

A consistently encountered effect of eserine on luminescence of extracts at low temperatures is illustrated in figure 14, namely, a strong tendency for recovery from the initial inhibition. The recovery sometimes amounted to more than a 10 fold increase in intensity. This phenomenon, whose interpretation is unclear, was not observed at the optimum temperature, and was much less prominent in the luminescence of whole cells (fig. 13).

Influence on oxygen consumption

Concentrations of DFP that reduced the intensity of luminescence by more than 90% at the optimum temperature did not reduce the rate of oxygen consumption of *A. fischeri* or *P. phosphoreum* cells, as measured by the "Dimming time" method, i.e., the length of time required for luminescence to disappear suddenly due to the exhaustion of the dissolved oxygen. In some instances, inhibition of luminescence was accompanied by an actual increase in rate of total oxygen consumption, by as much as 33%. Similar differences in the effects of Sarin on luminescence and total respiration, respectively, were found with both cells and extracts of *A. fischeri*. These results are in accord with previous observations of the influence of various narcotics on bacterial luminescence (Taylor, '34; van Schouwenburg, '38).

DISCUSSION

The experiments reported in this paper reveal that the net effect of DFP on the luminescent oxidative system of

luminous bacteria involves at least three types of reactions: (1), in an initial, rapid inhibition, an equilibrium combination of the drug with an essential enzyme (or enzymes), which is readily dissociated by dilution and partially dissociated by a rise in temperature from a cold to approximately optimum temperature; (2), a gradual reversing of inhibition at relatively low concentrations, especially evident at low temperatures; and (3), a progressively increasing, apparently irreversible inhibition at relatively high drug concentrations, through a reaction or reactions having a very high temperature coefficient.

The type of action wherein a drug has the dual effect of causing a readily reversible inhibition and also catalysis of an irreversible denaturation of the system, to extents dependent upon both temperature and concentration of the drug, is not uncommon ("Type II," Johnson et al., '54), though in the examples which have been partially analyzed there is little evidence for a greater amount of inhibition by a given concentration at a low temperature, comparable to that observed with DFP in the present study. A dual inhibitory effect, wherein both the reversible and progressively increasing inhibitions are enhanced by rise in temperature, has been reported by Nachmansohn, Rothenberg and Feld ('47) for the action of DFP, in concentrations of the order of 10^{-6} M, on cholinesterase from the electric organ of *Electrophorus electricus*. Their data indicate that the progressively increasing inhibition proceeds with the kinetics of a first order reaction (Johnson et al., '54), and this reaction possibly involves phosphorylation of the enzyme to a relatively stable, catalytically inactive product analogous to the product, which has been crystallized (cf. Balls and Jansen, '52) resulting from the stoichiometric reaction between DFP and chymotrypsin. The possibility is not excluded that a similar mechanism is involved in the highly temperature-sensitive, irreversible inhibitions of luminescence by DFP and Sarin in the present study, especially in view of the wide variations in velocity constants of the reactions between these drugs and

various enzymes at constant temperature (Michel, '55), suggestive of considerable variation also in energies of activation. Unfortunately, data with respect to the influence of temperature on the action of these drugs on various systems are only sparsely available. Further information, however, should help elucidate certain aspects of the mechanism of action, not only on enzyme systems *in vitro*, but possibly also on physiological effects *in vivo*, e.g., the resistance of bullfrogs to Sarin toxicity (Wilber, '54).

In regard to the tendency for the amount of inhibition to diminish with time, especially with moderate concentrations of the drugs at low temperatures, the evidence is not sufficient to suggest the most likely interpretation. It could merely represent a transitory change between different steady states of luminescence, such as follows the addition of acid (Johnson et al., '45). On the other hand, it might involve mechanisms similar to those responsible for gradual recovery from DFP poisoning of other living organisms. The tendency for rapid recovery of luminescence in extracts, from the initial inhibition caused by isopropyl alcohol at low temperatures, possibly involves a partial elimination of this drug through enzyme action.

A distinctly unusual feature of the action of DFP and Sarin found in the present study is the rapid, readily reversible initial inhibition of luminescence, and its independence of cell density. The concentrations involved, viz., of the order of 10^{-4} to 10^{-2} molar, are much higher than those required for corresponding amounts of inhibition of cholinesterases. On the other hand, they are within the range of concentrations effective for various dehydrogenases (Michaelis et al., '49). Atropine, in concentrations 10 or more times greater than DFP, did not counteract the DFP inhibition as it does in animals (Grob et al., '47) but added slightly to the amount of inhibition of luminescence. The action on luminescence appears to be of a general, "non-specific" type resembling that of eserine and a variety of narcotics to which the

luminescent system is sensitive, and much more so than other processes such as aerobic respiration in the same organisms.

SUMMARY

The action of di-isopropylfluorophosphonate (DFP), Sarin, eserine, fluoride and isopropyl alcohol on bacterial luminescence has been studied in relation to drug concentrations, temperature, and reversibility by dilution.

Luminescence of whole cells of *Achromobacter fischeri*, *A. harveyi*, and *Photobacterium phosphoreum* at the respective optimum temperatures is inhibited by 10^{-4} to 10^{-2} molar DFP in proportion to the first power of drug concentration, in a manner that is quantitatively reversible on dilution and independent of cell density.

The influence of temperature reveals that the net effect of DFP on the luminescence of whole cells involves three types of reaction: (1), a mobile equilibrium, responsible for a rapid, initial inhibition, in which the drug combines in a 1:1 ratio with an essential enzyme, and dissociates with rise in temperature or dilution; (2), a reaction through which a gradual recovery from the initial inhibition by the lower concentrations takes place, especially at low temperatures; and (3), an essentially irreversible reaction caused by the higher concentrations and greatly enhanced by a rise in temperature.

Sarin inhibits the luminescence of whole cells in the same range of molar concentrations as DFP and in essentially the same manner, but the irreversible reaction at the higher concentrations is more strongly temperature dependent.

Both DFP and Sarin inhibit the luminescence of cell-free, crude enzyme extracts. With Sarin, the same range of molar concentrations is required as for whole cells, whereas with DFP considerably stronger concentrations are required for inhibiting the luminescence of extracts. With the *in vitro* system, the initial inhibitory reaction, which is favored by low temperatures in whole cells, was only slightly evident.

The sustained luminescence of the extracted system is inhibited 30 to 60% by 0.011 M DFP with concentrations of each

of the following diffusible factors reduced, one at a time, to a level limiting the intensity of emitted light: flavine mononucleotide, decaldehyde, reduced diphosphopyridine nucleotide, and diphosphopyridine nucleotide plus malate.

The rate of total oxygen consumption in either cells or extracts of *A. fischeri* is not reduced, or is slightly increased, by DFP and Sarin, respectively, in concentrations that greatly inhibit the intensity of luminescence.

Eserine reversibly inhibits the luminescence of whole cells, in a range of concentrations nearly the same as that of DFP. The amount of inhibition is slightly increased on changing from a low temperature to the optimum temperature. With whole cells at a low temperature, there is a slight tendency for the inhibition to lessen with time, whereas with extracts this "escape" is rapid; the same concentration at the optimum temperature, however, causes a progressively increasing inhibition.

Relatively high concentrations of fluoride, of the order of tenths molar, are required to inhibit the luminescence of either cells or extracts, and still higher concentrations of isopropyl alcohol are required. The latter, in a concentration of 1 molar, causes an increase in intensity at low temperatures, but a pronounced and rapidly increasing inhibition at the optimum temperature.

ACKNOWLEDGMENTS

The authors acknowledge with pleasure and appreciation the interest and cooperation of the Army Chemical Center, where all the experiments with DFP and Sarin described in this paper were carried out. Gratefulness is expressed in particular, to Colonel Elton and Dr. Bruce Dill for their permission to work at the Center, and likewise to Dr. Amedeo Marrazzi, who with Dr. Ross Hart, Dr. Penneys, and other members of the staff of the Clinical Research Division, substantially aided the course of this investigation.

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COMMENTS AND COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

2,3-DIPHOSPHOGLYCERATE AND OTHER INTRA-ERYTHROCYTIC ACID SOLUBLE PHOSPHATES IN YOUNG RABBITS¹

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TWO FIGURES

The experimental production of erythroblastosis fetalis in rabbits has been reported by Kellner and Hedal ('53) and by Anderson ('56). In connection with studies in progress in our laboratories on the erythrocytes of infants with hemolytic disease of the newborn (Greenwalt and Ayers, '56), the availability of test animals with this syndrome is desirable. Phosphate partition studies on erythrocytes of the newborn rabbit for comparison with the human have therefore been conducted. The 2,3-DPG² component of the red cells of normal newborn rabbits was found to be less than one-third the

¹ This investigation was supported by a grant from the Edward A. Uhrig Foundation.

² The following abbreviations are used: 2,3-DPG, P_i, ATP and ADP for 2,3-diphosphoglycerate, inorganic orthophosphate, adenosine-5'-triphosphate and adenosine-5'-diphosphate respectively.

amount found in the human at birth. It increases about five-fold to adult rabbit levels during the first few weeks of growth.

MATERIALS AND METHODS

Rabbits less than 8 hours of age to three weeks of age were studied. The newborn animals constituted the largest group (42 rabbits). Groups from 6 to 20 days old were also examined (1 to 6 rabbits per group) and adult rabbits were included for comparison.

Each ml of blood obtained by heart puncture was mixed with 0.15 ml of standard acid-citrate-dextrose solution and promptly placed in an ice-bath.³ Preparation of a neutralized perchloric acid filtrate (Neuberg, Strauss and Lipkin, '44) was conducted at 4°C as follows: After removal of the plasma the red cells were washed once with saline. A 50% suspension was prepared and an aliquot was mixed with an equal volume of 10% perchloric acid. The supernatant was neutralized to pH 7.0 with KOH, centrifuged and the clear extract stored at -20°C until analyzed.

ATP, ADP and 2,3-DPG were separated by two-dimensional paper chromatography at room temperature on Whatman no. 4 paper. Aliquots of 200 μ l were developed for 24 hours in a descending system of n-propanol/ammonia/water, 60/30/10 (Caldwell, '53) followed by 16 hours in an ascending system of methanol/formic acid/water, 80/15/5 (Bandurski and Axelrod, '51). The ATP and ADP spots were located visually by U. V. quenching, identity being established by comparison with reference samples (Pabst Laboratories⁴) and by their characteristic spectrophotometric constants (Bock, Ling, Morell and Lipton, '56). The 2,3-DPG spot was detected by spraying successively with alcoholic solutions of ferric chloride and sulfosalicylic acid (Wade and Morgan, '53), its identity being established by comparison with a reference sam-

³ The 14 and 16-day samples (table 1) were incubated at 37°C for 15 minutes prior to preparing the perchloric acid filtrates.

⁴ We wish to thank Pabst Laboratories for the reference samples of ATP and ADP.

TABLE 1
Effect of age on the acid-soluble phosphates in rabbit erythrocytes

AGE	NO. TESTED	RETICU- LOCYTES	$\mu\text{M P}/100 \text{ ML OF PACKED CELLS}^1$					Total P
			2,3-DPG	P _i	ATP	ADP		
<i>days</i>		%						
0 (< 8 hrs.)	42	26	179 (92-274)	175 (47-315)	414 (218-564)	86 (5-147)	1179 (984-1407)	
6	3	13	561 (532-577)	137 (105-156)	512 (451-564)	95 (63-149)	1505 (1395-1617)	
8	1	16 ²	535	171	487	94	1726	
13	2	11	853 (836-871)	131 (107-156)	454 (425-483)	74 (46-102)	1839 (1798-1881)	
14 ³	5	25 ²	706	253	243	102	1705	
16 ³	6	6	883	255	317	73	1971	
20	2	8	1161 (1093-1228)	79 (78-80)	560 (542-578)	65 (18-113)	1844 (1832-1856)	
Adult	2	—	957 (867-1046)	55 (41-68)	463 (394-531)	117 (101-133)	1858 (1848-1868)	
Adult	4	—	(581-755)	(161-226)	(65-113)	(48-81)	—	

¹ Numbers not in parentheses represent means, numbers in parentheses represent range.

² Previously bled.

³ Blood of litter mates pooled to obtain mean values.

⁴ Prankerd and Altman ('54).

ple (Schwarz Laboratories⁵). The identified areas were eluted with 0.1 N HCl for 18 hours and wet-ashed to orthophosphate for analysis. The neutralized perchloric acid filtrates were analyzed for inorganic phosphate (P_i) as described by Schaffer, Fong and Kirk ('53) and for total P by wet-ashing ($H_2SO_4-HClO_4$) to orthophosphate (P_i).

Duplicate chromatographic analysis of the neutralized perchloric acid filtrates showed a reproducibility within the limits of 5-10%. Recovery of the known reference compounds was 85-95%. For the total P determinations, the coefficient of variation was 2.02%.

RESULTS

The effect of age on the composition of the acid-soluble phosphates in rabbit erythrocytes is presented in table 1.

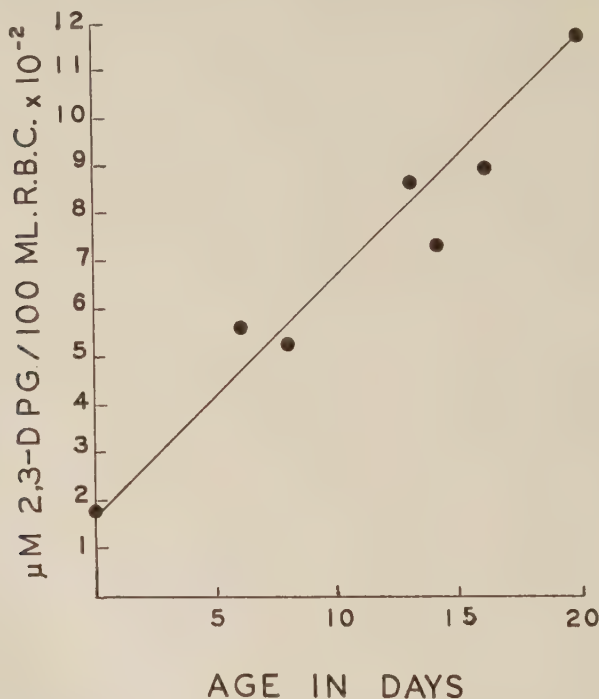


Fig. 1 2,3-DPG content of rabbit erythrocytes during first 20 days of growth.

⁵ We wish to thank Schwarz Laboratories for the reference sample of 2,3-DPG.

The data of Pranker and Altman ('54) for the adult rabbit are included. The average per cent reticulocytes for each age group is also given. The low 2,3-DPG content of the newborn rabbit (92–274 $\mu\text{M}/100$ ml red cells) attains the adult level (867–1046 $\mu\text{M}/100$ ml red cells) in about two to three weeks. This increase is approximately linear as shown in figure 1. The relatively higher P_i values found during the first few weeks are in keeping with previous findings in the young of other mammalian species (Guest and Rapoport, '39; Greenwalt and Ayers, '56). No significant differences in ATP and ADP were observed. The relatively high P_i and low ATP values found in the 14 and 16 day samples were probably a result of the incubation of these particular samples.³ The rise in total P during the first three weeks (1179–1844 $\mu\text{M}/100$ ml red cells) is accounted for by the 2,3-DPG increase (179–1161 $\mu\text{M}/100$ ml red cells). The average recovery of total P in terms of the compounds analyzed (2,3-DPG + P_i + ATP + ADP) was 80.2%.

DISCUSSION

Although the 2,3-DPG showed a linear rise with age, no correlation with the number of reticulocytes was demonstrable (fig. 2). Repeated bleeding of the same rabbit, with maintenance of higher reticulocyte counts, had no effect on the phosphate distribution. Spherocytosis was not observed. Low white cell counts in the original samples (1400–1600/mm³), as well as careful removal of the buffy layer formed on centrifuging, precluded any effects of leucocytes.

The 2,3-DPG increase in the rabbit erythrocyte during the newborn period is too rapid to be explained by a shift of hematopoiesis from extra-medullary to medullary sites. Although a clear-cut interpretation of the observed rise in erythrocyte 2,3-DPG with age is not indicated from the present data, the appearance or increase of an enzyme system essential for 2,3-DPG formation is suggested.

³ See footnote 3, page 308.

SUMMARY

The acid soluble phosphates of the erythrocytes of young rabbits were separated by two-dimensional chromatography. The 2,3-DPG content was found to be unexpectedly low at birth and increased five-fold to adult levels during the first three weeks of life. The rise of 2,3-DPG with age was linear.

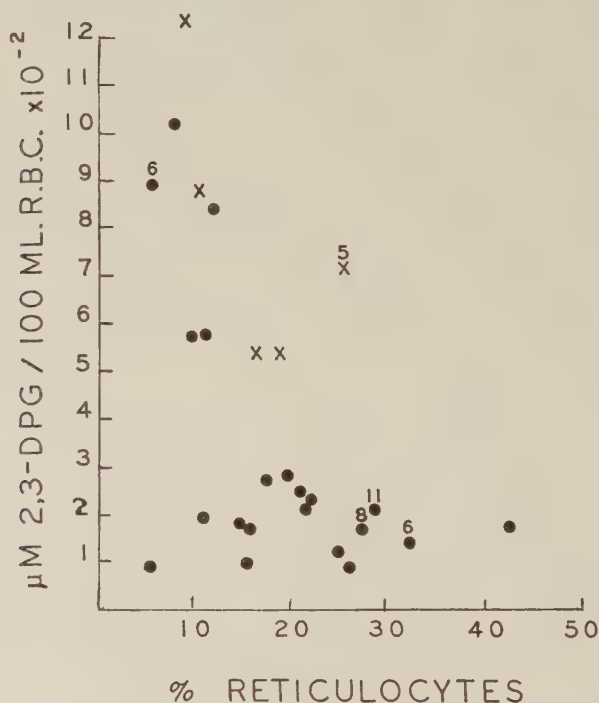


Fig. 2 Lack of correlation of intra-erythrocytic 2,3-DPG with reticulocytosis: ●, rabbits not previously bled; X, bled previously; numerals indicate number of individuals in pooled specimens. All values below 300 μ M are from newborn rabbits.

ACKNOWLEDGMENTS

The technical assistance of Peggy Hoffman and Mavis Moorman is gratefully acknowledged.

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SPECIES VARIATION IN THE ENZYME CONTENT
OF THE CORNEAL EPITHELIUM

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TWO FIGURES

Enzymatic studies of the rat cornea undertaken in this laboratory have revealed the corneal epithelium to contain many of the enzymes participating in carbohydrate metabolism. Lactic dehydrogenase activity was found to be several times higher than the activity of the other systems

present (Kuhlman and Resnik, '58). The levels of enzyme activity in the corneal epithelium approach those of the more active cell layers of brain (Lowry, Roberts, Wu, Hixon and Crawford, '54; Lowry, Roberts, Leiner, Wu, Farr and Albers, '54; Lowry, Roberts and Chang, '56) and the retina (Lowry, Roberts and Lewis, '56). The studies of deRoethth ('50, '51) and Herrmann and Hickman ('48b) indicate that the cornea has a very active metabolism which is localized in its epithelial and endothelial layers. The stroma, however, has little metabolic activity.

Except for minor variations, basically the cornea has the same general structure in all mammalian species. However, Virchow ('10) described species differences in the number of cell layers comprising the corneal epithelium; for example the horse has 20 layers, and the human has 6. In addition, species variation in the mitotic rate of the basal layer of the epithelium and the presence and absence of Bowman's membrane have been described (Pirie and van Heyningen, '56; Buschke, Friedenwald and Fleischman, '43; Duke-Elder, '37).

Since there is a morphological variation between species, there may be quantitative or qualitative differences in the enzyme content of the corneal epithelium. To investigate this question and to extend our knowledge of the enzyme content of the corneal epithelium, the following enzyme systems were assayed in several species: hexokinase, aldolase, and glucose-6-phosphate, isocitric, lactic and malic dehydrogenases, and glutathione reductase. In addition, the properties of lactic dehydrogenase were studied. Microchemical techniques were used to obtain samples of lyophilized corneal epithelium from the rat, cat and rabbit. An attempt is made to relate the activity of these enzymes to the physiology of the cornea.

METHODS AND MATERIALS

Rats (150 to 200 gm) of the Sprague-Dawley strain, white rabbits 12 weeks of age, and cats of unknown breed and age

were used. The animals were killed by cervical fracture and the eyes rapidly removed and frozen in liquid nitrogen. Frozen sections of the cornea were cut and lyophilized (Lowry, '53; Lowry, Roberts, Wu, Hixon and Crawford, '54). Material was stored two to three months at -20°C in evacuated containers. Areas of lyophilized epithelium weighing 1 to 2 μg were dissected and weighed on the Lowry quartz fiber balance (Lowry, '41, '44; and Anfinsen, Lowry and Hastings, '42).

Lactic, malic and glucose-6-phosphate dehydrogenases and aldolase were assayed by established procedures (Lowry, Roberts, Wu, Hixon and Crawford, '54; Lowry, Roberts, Wu, Leiner, Farr and Albers, '54; Lowry, Roberts and Lewis, '56; Robins, '57; Kuhlman and Lowry, '56; Lowry, '57) or by modifications to be discussed (see table 1). Hexokinase and isocitric dehydrogenase were assayed by methods of Lowry, as yet unpublished. Glutathione reductase was assayed by a method developed in this laboratory (Kuhlman and Resnik, '58).

RESULTS

Characteristics of lactic dehydrogenase

When a 1.5 mM concentration of pyruvate was used in the assay of rabbit corneal epithelium lactic dehydrogenase activity, the results were dependent upon sample size (fig. 1). This suggested that the amount of substrate present was insufficient for assay of high activity samples. When a higher concentration of substrate was used an average activity of 1480 MKH was obtained and no dependence upon sample size was evident. The values obtained for rat and cat corneal epithelium (table 2) were a fraction of this amount, and raised the question of a species variation in the characteristics of lactic dehydrogenase. Consequently, the pH optimum and Michaelis constants for lactic dehydrogenase were determined for the three species.

TABLE 1

Summary of substrate reagents used in the assay systems

ENZYME	HEXOKINASE	ALDOLASE	GLUCOSE-6-PO ₄ DEHYDROGENASE	LACTIC DEHYDROGENASE	MALIC DEHYDROGENASE	ISOCITRIC DEHYDROGENASE	GLUTATHIONE REDUCTASE
pH	8.10	8.70	9.45	8.75	8.74	8.10	7.20
Buffer	TRIS	Hydrazine	AMP ₂	TRIS	TRIS	TRIS	Phosphate
Substrate	Glucose	Fructose-1-6 di PO ₄	Disodium glucose-6-PO ₄	Sodium pyruvate	Sodium oxaloacetate	Sodium iso-citrate	Oxidized glutathione
Other factors	ATP KH ₂ PO ₄ Mg ⁺⁺ Zwischenferment TPN ⁺	NONE	TPN ⁺ EDTA Mg ⁺⁺	DPNH Nicotinamide	DPNH Nicotinamide	TPN ⁺ Mn ⁺⁺ Nicotinamide	TPNH EDTA Nicotinamide
Product measured	TPNH	Glyceraldehyde-3-PO ₄	TPNH	DPN ⁺	DPN ⁺	TPNH	TPN ⁺

TRIS = Tris. (hydroxy-methyl)-amino-methane.

AMP₂ = 2-amino-2-methyl-1,3-propanediol.

TPN⁺, DPN⁺, TPNH, DPNH = oxidized and reduced tri- and di-phosphopyridine nucleotide.

ATP = Adenosine triphosphate.

EDTA = ethylene diamine tetra acetate.

The pH optimum at a pyruvate concentration of 4.4 mM was found in all three cases to be near pH 8.75 (fig. 2). When a substrate concentration of 0.5 mM was used, the pH optimum was in accord with the published value of 7.40. The Michaelis constants (K_m) for lactic dehydrogenase from

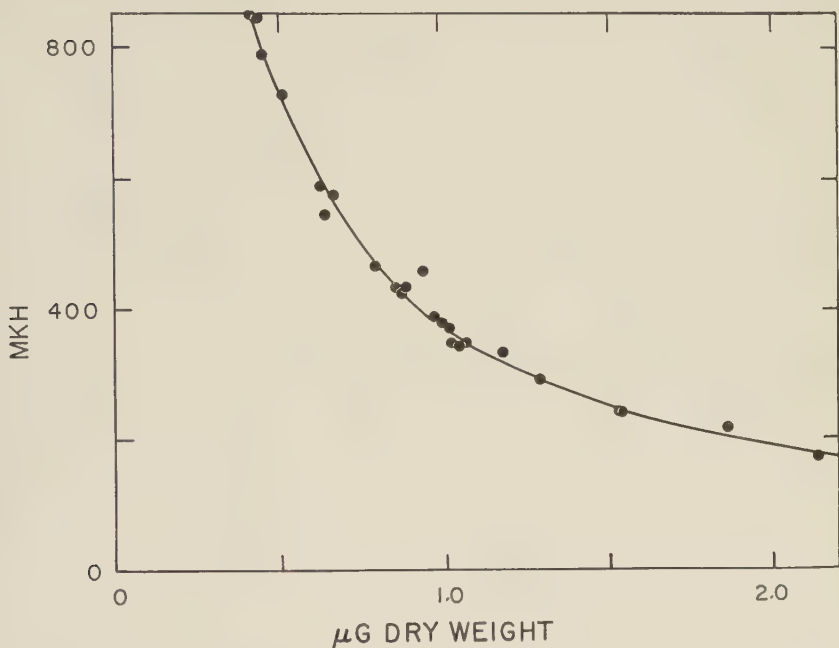


Fig. 1 The dependence of lactic dehydrogenase activity upon sample weight. Activity is expressed as moles of DPN^+ produced in the reduction of pyruvate, per kilogram dry weight of rabbit corneal epithelium per hour. See text for explanation. The system contained 300 μl of substrate prepared at pH 7.40 with a pyruvate concentration of 1.5 mM.

each of the three species were determined at 33.6°C (see table 3).

In summary, the pH optimum was similarly dependent on substrate concentration in all three instances. At pH 8.75 the Michaelis constants, within the limits of experimental error, are essentially equal. The value for rabbit LDH at pH 7.40 is slightly higher than the value of $5.2 \times 10^{-5}\text{M}$ for

TABLE 2
Enzyme activity in the corneal epithelium of the rabbit, cat and rat

	LDH	GSSGH	ALDOLASE	G6DH	HK	MDH	ICDH
Rabbit	2150 ¹	1.39 ¹	0.345 ¹	4.12 ¹	0.848 ¹	85.9 ¹	9.34 ¹
n ²	12	37	5	7	48	7	41
s.e.m.	± 60.4	± 0.0768	± 0.0355	± 0.633	± 0.0401	± 1.39	± 0.408
Cat	103.	1.65	0.678	2.11	0.760	51.1	2.80
n	18	6	3	4	7	11	5
s.e.m.	± 8.93	± 0.248	± 0.0190	± 0.503	± 0.0604	± 2.52	± 0.214
Rat	87.4	1.50	0.330	2.32	0.209	62.5	4.26
n	13	4	3	5	9	12	13
s.e.m.	± 4.38	± 0.280	± 0.0486	± 0.795	± 0.0395	± 3.01	± 0.478

¹ Activity expressed as moles of substrate split or oxidized per kilogram dry weight of corneal epithelium per hour.

² "n" represents the number of determinations in each case. Three cats, 5 rats and 10 rabbits provided all the corneal specimens.

The following abbreviations are used: LDH, lactic dehydrogenase; GSSGH, glutathione reductase; G6DH, glucose-6-phosphate dehydrogenase; HK, hexokinase; MDH, malic dehydrogenase; ICDH, isocitric dehydrogenase; s.e.m., standard error of mean.

pig heart LDH (Colowick and Kaplan, '55). The K_m of rabbit LDH is smaller at pH 7.40 than at 8.75. These findings fail to indicate significant species difference in the characteristics of lactic dehydrogenase.

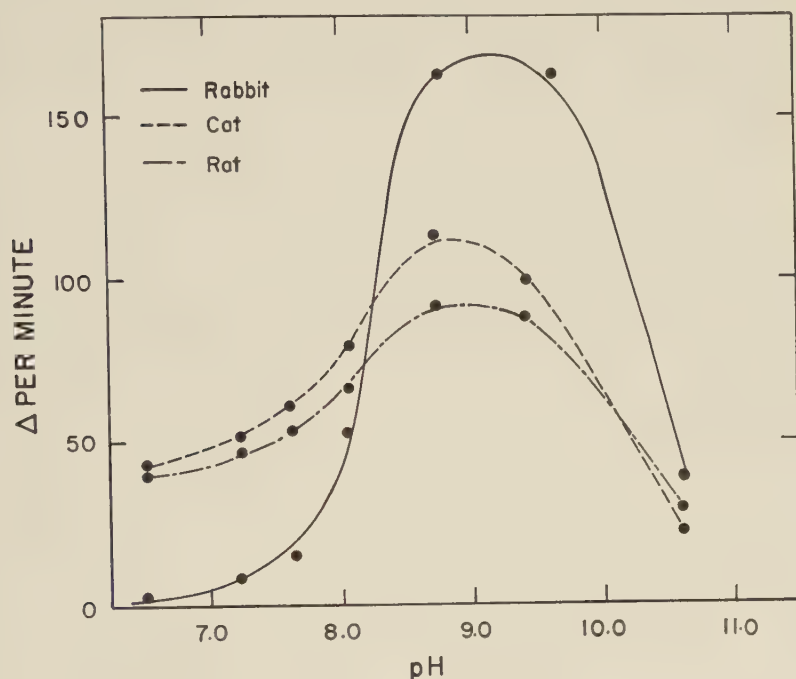


Fig. 2 The pH optimum at a pyruvate concentration of 4.4 mM for lactic dehydrogenase from the cornea of rat, cat and rabbit at 33.6°C. Activity is expressed as change of optical density per minute at 340 $m\mu$. Rabbit values are represented 1/20 scale. Each cuvette contained 504 μ l of substrate of the following composition: buffer 0.1 M, pyruvate 4.4 mM, and DPNH 2 mM. An aliquote of 2.41 μ l of rat or cat and 0.928 μ l of rabbit corneal extract was added to start the reaction in each case. The extract was prepared by crushing the corneas in 10 times their weight of cold water.

TABLE 3

Michaelis constants for LDH of cat, rat and rabbit corneal epithelium at 33.6°C

	pH 7.40	pH 8.75
Cat	—	2.7×10^{-3} M
Rat	—	1.8×10^{-3} M
Rabbit	1.7×10^{-4} M	1.4×10^{-3} M

Enzyme measurements

Utilizing these observations, lactic dehydrogenase was re-assayed with a high (4.0 mM) level of pyruvate at pH 8.75. A final value of 2150 MKH was obtained for rabbit corneal epithelium. Samples of 0.3 to 1.5 μ g were used and diluted 5 to 10×10^5 times with substrate to minimize the influence of tissue inhibitors. No dependence of activity upon sample size was noted.

The level of lactic dehydrogenase activity in the rabbit corneal epithelium is 5 to 10 times greater than has been previously reported for tissue assay of any enzyme. (Compare table 2 and table 4). Comparison with lactic dehydrogenase assays of carcinomas and sarcomas indicate that even they do not have a level of activity near that of rabbit corneal epithelium (Meister, '50). The activity of lyophilized rabbit corneal epithelium is comparable to a 1:10 to 1:50 dilution of a commercial preparation of crystalline lactic dehydrogenase.¹ This extremely high LDH activity is peculiar to rabbit as the cat and rat have only 1/20 the activity. The fact that the K_m and pH optimum for lactic dehydrogenase of these species is the same indicates the above results are not the consequence of a species difference in the characteristics of the enzyme.

Malic dehydrogenase activity, though highest in rabbit corneal epithelium, is also high in the other species and is of the same order of magnitude in all cases. Both lactic and malic dehydrogenase appear to be much more active when assayed separately than are the over-all cycles in which they participate. In the cellular areas of brain, malic dehydrogenase is more active than LDH (Strominger and Lowry, '55) but such is not the case in corneal epithelium. In the rabbit corneal epithelium particularly, LDH is 25 times more active than malic dehydrogenase.

Isocitric dehydrogenase activity is most prominent in rabbit and lowest in the cat corneal epithelium. The abundancy

¹ Obtained from Sigma Chemical Company, St. Louis, Missouri.

Comparison of enzyme activity¹ in other locations

	MDH	LDH	ALDOLASE	GDH	HK	G6DH	IDDH
Cerebral cortex (monkey) ²							
Layer							
1	79.8	23.8	6.34	0.686	—	—	—
3	84.0	21.4	6.27	0.493	—	—	—
5	69.5	22.3	6.04	0.347	—	—	—
7	22.4	12.4	2.69	0.187	—	—	—
Cerebellar cortex (monkey) ³							
Molecular layer	34.8	10.7	9.25	—	—	—	—
Granular layer	29.9	9.7	5.65	—	—	—	—
White matter	10.0	4.5	3.45	—	—	—	—
Retina (rabbit) ⁴							
Layer							
2b	138.	24.4	—	—	—	—	—
4	40.6	52.9	—	—	—	—	—
8	20.4	97.6	—	—	—	—	—
Single cells (monkey) ⁵							
Anterior horn cell	264	32	—	3.6	6.3	0.98	5.1
Ganglion cell bodies	231	50	—	2.5	7.2	2.15	4.5
Radiata (layer)	342	89	—	3.7	12.5	1.45	9.9
Cerebral cortex (rat) ⁶							
Layer							
1	65.6	42.3	—	4.79	—	1.48	—
3	66.8	37.3	—	3.45	—	1.51	—
5	51.4	27.8	—	3.06	—	1.16	—
7	7.50	22.6	—	1.09	—	0.866	—

¹ Activity is expressed as moles of substrate utilized per kilogram fat free dry weight of tissue per hour (MKH). LDH and MDH activity in cases 2, 3, 4, and 6 are measured in the direction of DPNH formation. The velocity in the direct comparable to the cornea data is approximately 4 and 5 times the above values respectively.

GDH = glutamic dehydrogenase.

² Robins, Smith, Eydt and McCaman, '56.

³ Robins and Smith, '53.

⁴ Lowry, Roberts and Lewis, '56.

⁵ Lowry, '57.

⁶ Kuhlman and Lowry, '56.

of ICDH and MDH would suggest that the citric acid cycle may be important in corneal metabolism.

Glucose-6-phosphate dehydrogenase has highest activity in rabbit and lower activity in rat and cat corneal epithelium. Significant *zwischenferment* activity would be expected in view of the importance of the direct oxidative pathway of metabolism in the cornea. (Kinoshita and Masurat, '54; Kinoshita, Masurat and Helfant, '55).

Minor variation was noted in hexokinase and aldolase content, while glutathione reductase activity remained the same in all these species.

DISCUSSION

Generally the corneal epithelium of the rabbit has greater enzyme activity than that of the cat or rat, but this is particularly true in the case of LDH. Of interest is the fact that, of the three species, only the rabbit does not have Bowman's membrane beneath its corneal epithelium. One is tempted to relate this very high LDH activity in some way to the more important energy requiring functions of the cornea, such as maintenance of dehydration and the regenerative capacity of its epithelium.

The presence of large amounts of lactic dehydrogenase in the corneal epithelium bears a relationship to several previously unexplained observations which have been made concerning corneal metabolism. Herrmann and Hickman ('48b) have noted that the high lactate concentration present in the cornea decreases when it is incubated in a medium free of glucose. Pyruvate itself has never been found in appreciable quantities in the cornea. Pyruvate, when injected into the corneal stroma, rapidly disappears, indicating that it is either rapidly metabolized through the tricarboxylic acid cycle or converted to lactate (Herrmann and Hickman, '48a). In the absence of oxygen all three layers of the cornea can form lactate at a rapid rate, but in the presence of oxygen the whole cornea has been shown by deRoethth ('51) to produce no more

lactate than could be accounted for by the stroma alone. These observations indicate the corneal epithelium produces virtually no lactate in the presence of oxygen. Malignant tumors which, on the other hand, also have large amounts of lactic dehydrogenase are characterized by aerobic glycolysis (Warburg, Posener and Negelein, '24; Cori and Cori, '25).

Corneas of guinea pigs fitted with non-ventilated contact lenses show a marked rise in lactate content and become edematous (Smelser and Chen, '53; Smelser, '52; Langham, '52). The same changes take place when the cornea is placed in a nitrogen atmosphere. Under such conditions the basal layers of the epithelium show depletion of their normally rich glycogen stores (Smelser and Ozanics, '53). The lactate content of the cornea does not rise when the lids of an animal are sutured closed for periods less than 4 hours, and this has been explained by the diffusion of oxygen from the lid vessels (Langham, '52).

Attempting to integrate these findings with the high lactic dehydrogenase content of the cornea, as well as with the rest of its general enzymatic character, one may postulate that the cornea is able to utilize lactate from its surroundings. The anterior portion of the cornea is bathed in tear fluid and the posterior portion with the aqueous humor. Both these fluids contain an abundant supply of lactate (Pirie and van Heyningen, '56; Becker and Constant, '56). Lactic dehydrogenase, in the presence of DPN^+ , could convert this lactate to pyruvate, whereupon it could enter the citric acid cycle and be metabolized aerobically with subsequent production of high energy phosphate bonds. There is histochemical evidence for the presence of the cytochrome system in the corneal epithelium (Pirie and van Heyningen, '56).

Although, in the absence of oxygen, glucose and glycogen stores could be mobilized through the Embden-Meyerhoff cycle and converted to lactate, this pathway is less efficient in the production of ATP than the aerobic route. Since anaerobic conditions result in edema of the cornea, it appears that the

anaerobic glycolytic pathway fails to produce enough energy to enable the cornea to maintain its dehydrated state.

It seems likely at this point that the cornea uses lactate and that aerobic systems are important to the maintenance of corneal dehydration. These factors may also be important for the high mitotic rate of the corneal epithelium.

SUMMARY

Malic, lactic, glucose-6-phosphate, and isocitric dehydrogenases, hexokinase, aldolase and glutathione reductase were assayed in samples of lyophilized corneal epithelium from the cat, rat and rabbit. Rabbit corneal epithelium was found to have a very high level of lactic dehydrogenase, which was only 1/20 as high in the cat and rat. All three species had grossly similar levels of the other enzymes investigated. The enzymatic characteristics of corneal epithelium are discussed in relation to physiological observations made in the past by other workers.

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